

University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

Dissertations, Theses, & Student Research in
Food Science and Technology

Food Science and Technology Department

Fall 9-19-2011

Effect of Sodium Nitrite, Sodium Erythorbate and Organic Acid Salts on Germination and Outgrowth of *Clostridium perfringens* Spores in Ham during Abusive Cooling

Mauricio A. Redondo

University of Nebraska-Lincoln, hiequel@gmail.com

Follow this and additional works at: <https://digitalcommons.unl.edu/foodscidiss>



Part of the [Food Chemistry Commons](#), [Food Microbiology Commons](#), and the [Food Processing Commons](#)

Redondo, Mauricio A., "Effect of Sodium Nitrite, Sodium Erythorbate and Organic Acid Salts on Germination and Outgrowth of *Clostridium perfringens* Spores in Ham during Abusive Cooling" (2011). *Dissertations, Theses, & Student Research in Food Science and Technology*. 18.
<https://digitalcommons.unl.edu/foodscidiss/18>

This Article is brought to you for free and open access by the Food Science and Technology Department at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Dissertations, Theses, & Student Research in Food Science and Technology by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

EFFECT OF SODIUM NITRITE, SODIUM ERYTHORBATE AND
ORGANIC ACID SALTS ON GERMINATION AND OUTGROWTH OF
CLOSTRIDIUM PERFRINGENS SPORES IN HAM DURING ABUSIVE
COOLING

By

Mauricio Redondo-Solano

A THESIS

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Master of Science

Major: Food Science and Technology

Under the supervision of Professor Harshavardhan Thippareddi

Lincoln, Nebraska

September, 2011

EFFECT OF SODIUM NITRITE, SODIUM ERYTHORBATE AND ORGANIC ACID
SALTS ON GERMINATION AND OUTGROWTH OF *CLOSTRIDIUM*
PERFRINGENS SPORES IN HAM DURING ABUSIVE COOLING

Mauricio Redondo-Solano, M. S.

University of Nebraska, 2011

Advisor: Harshavardhan Thippareddi

The effect of sodium nitrite (NaNO_2), sodium erythorbate, sodium chloride (NaCl) and organic acid salts (potassium lactate and sodium diacetate) on *C. perfringens* spore germination and outgrowth in ham was evaluated. The research was divided in two parts. The first experiment consisted of potential *C. perfringens* spore germination and outgrowth in ham containing combinations of NaNO_2 (0, 50, 100, 150 or 200 ppm) and sodium erythorbate (0 or 557 ppm) during cooling. This experiment included the evaluation of residual nitrite levels as affected by temperature (heat shock at 75°C for 20 min and subsequent cooling from 54.4°C to 4.4°C within 15 h), storage time (3 and 24 h at 5°C) and sodium erythorbate concentration. Inhibition of *C. perfringens* spore germination and outgrowth was observed after 3 h of ham preparation in all the treatments evaluated. Greater inhibition of *C. perfringens* was observed with higher concentrations of NaNO_2 . Addition of sodium erythorbate resulted in greater *C. perfringens* populations subsequent to 15 h of abusive cooling. Residual nitrite concentrations were similar under the experimental conditions used in this study. Residual oxygen in the ham could explain the inhibition observed during cooling of

the ham within 3 h of preparation. During the second experiment, the effect of NaNO₂ (0 or 100 ppm), NaCl (1 or 2%) and organic acid salts (*Opti.Form* PD4®; 0, 1.5 or 2.5%) on *C. perfringens* spore germination and outgrowth during abusive cooling was evaluated. Incorporation of *Opti.Form* PD4® (1.5%) inhibited *C. perfringens* spore germination and outgrowth regardless of the cooling rate (9, 15 or 21 h). Addition of NaNO₂ enhanced the antimicrobial activity of organic acid salts. Shorter time period between ham preparation and heat treatment/cooling resulted in greater inhibition of *C. perfringens* spore germination and outgrowth. Antimicrobial agents should be incorporated into ham formulations in case reductions in NaCl and NaNO₂ concentrations are considered in view of the recommendation to reduce sodium content in the diet.

ACKNOWLEDGMENTS

In the first place I would like to thank God for the blessings I have received in my life. In second place I must thank my mother and sisters for their support through my entire journey during my academic life. To my mother I owe everything I am and everything I have accomplished. This work is especially dedicated to her. For Carol, my wife, I do not have enough words to express the importance of her presence in my life and how important she was for finishing this project. The real meaning of this accomplishment has Carol's name and all my Master's experience made sense from the beginning because of her. A special mention to my advisor Dr. Harshavardhan Thippareddi, who gave me the opportunity to be part of the UNL family and has helped me to expand my professional criteria and scientific perspective. He knows that, after accepting me as a student, he changed my life in many ways. Finally, I would like to thank the University of Costa Rica and my friends in there for the continuous support and opportunities during the last 3 years.

TABLE OF CONTENTS

List of Figures	vi
List of Tables.....	viii
Introduction	ix
 I. CHAPTER 1.....	 1
Literature review	2
 1. <i>Clostridium perfringens</i>	 2
1.1. General characteristics.....	3
1.2. Toxin production by <i>C. perfringens</i>	4
1.3. Occurrence of disease and pathogenesis	5
1.4. Historic background and current health impact.....	8
1.5. Implicated foods and control	10
 2. Sodium nitrite for curing and preservation of meat products	 13
2.1. General information.....	13
2.2. Historic background of meat curing	14
2.3. The chemical process of meat curing	16
2.4. Antimicrobial aspects of nitrites in cured products	18
2.5. <i>C. perfringens</i> sensitivity to nitrites	21
2.6. Nitrite regulation in meat products and health concerns	23
2.7. Presence of sodium erythorbate in cured products	27
 3. Determination of nitrite in cured meat products	 28
3.1. Presence of nitrite in meat products	28

3.2. Monitoring of nitrite in cured products	32
3.3. Technical considerations for analysis of nitrites in cured meats	33
3.4. Analytical methods for nitrites in cured meats	35
3.5. High Performance Liquid Chromatography (HPLC) for nitrites in cured meats	37
4. Reduction of sodium content in meat products.....	41
4.1. General background.....	42
4.2. Metabolic activityof sodium.....	44
4.3. Excessive sodium intake and high blood pressure	45
4.4. Salt, an ingredient with powerful technical functions for meat products	50
4.5. Antimicrobial properties of salt.....	53
4.6. Microbiological studies in reduced salt products	56
5. Use of organic acids and their salts for food preservation	57
5.1. General information.....	57
5.2. Regulation and health concerns.....	59
5.3. Applications of organic acids and their salts in foods	60
5.4. Antimicrobial activity of organic acids and their salts	61
5.5. Scientific studies involving organic acids and their salts	64
6. References	67

II. CHAPTER 2.....	84
7. Effect of Sodium Nitrite and Sodium Erythorbate on Germination and Outgrowth of <i>Clostridium perfringens</i> Spores in Ham During Abusive Cooling.....	85
7.1. Abstract.....	85
7.2. Introduction	86
7.3. Materials and methods.....	87
7.4. Results and discussion	91
7.5. Conclusions	99
7.6. References	100
7.7. List of Tables	104
7.8. Legend to the Figures	105
III. CHAPTER 3.....	110
8. Control of <i>Clostridium perfringens</i> Spore Germination and Outgrowth by Potassium Lactate and Sodium Diacetate in Ham Containing Reduced Sodium Chloride	111
8.1. Abstract.....	111
8.2. Introduction	112
8.3. Materials and methods.....	114
8.4. Results and discussion	117
8.5. Conclusions	124
8.6. References	125
8.7. List of Tables	128
8.8. Legend to the Figures	129

IV. CHAPTER 4.....	135
9. Recommendations for future research.....	136
APPENDIX.....	137
A.1 Mean <i>C. perfringens</i> populations (\pm SD) in ham formulated with different concentrations of NaNO ₂ and cooled from 54.4°C to 4.4°C within 15 h subsequent to 3 h of storage at 5°C after preparation.....	138
A.2 Mean <i>C. perfringens</i> populations (\pm SD) in ham formulated with different concentrations of NaNO ₂ and cooled from 54.4°C to 4.4°C within 15 h subsequent to 24 h of storage at 5°C after preparation.....	139
A.3 Mean <i>C. perfringens</i> populations (\pm SD) in ham formulated with different concentrations of NaCl and cooled from 54.4°C to 4.4°C within 9 h subsequent to 3 h of storage at 5°C after preparation.....	140
A.4 Mean <i>C. perfringens</i> populations (\pm SD) in ham formulated with different concentrations of NaCl and cooled from 54.4°C to 4.4°C within 15 h subsequent to 3 h of storage at 5°C after preparation.....	141
A.5 Mean <i>C. perfringens</i> populations (\pm SD) in ham formulated with different concentrations of NaCl and cooled from 54.4°C to 4.4°C within 21 h subsequent to 3 h of storage at 5°C after preparation.....	142
A.6 Mean <i>C. perfringens</i> populations (\pm SD) in ham formulated with different concentrations of NaCl and cooled from 54.4°C to 4.4°C within 9 h subsequent to 24 h of storage at 5°C after preparation.....	143
A.7 Mean <i>C. perfringens</i> populations (\pm SD) in ham formulated with different concentrations of NaCl and cooled from 54.4°C to 4.4°C within 15 h subsequent to 24 h of storage at 5°C after preparation.....	144

A.8 Mean <i>C. perfringens</i> populations (\pm SD) in ham formulated with different concentrations of NaCl and cooled from 54.4°C to 4.4°C within 21 h subsequent to 24 h of storage at 5°C after preparation.....	145
A.9 Formulation for ham (control) prepared to evaluate the effect of different NaNO ₂ and sodium erythorbate levels on <i>C. perfringens</i> spore germination and outgrowth during abusive cooling (Chapter 2)	146
A.10 Formulation for ham (control) prepared to evaluate the effect of different levels of NaNO ₂ , NaCl and <i>Opti.Form</i> PD4® on <i>C. perfringens</i> spore germination and outgrowth during abusive cooling (Chapter 3)	147
A. Fig. 1 Temperature profiles for cooling of hams from 54.4°C to 4.4°C within 9,15 or 21 h.	148

LIST OF FIGURES

Fig.1.1. Pathogenesis of Type A foodborne illness by <i>C. perfringens</i> enterotoxin (CPE)	8
Fig.1.2. Molecular structures of erythorbic and ascorbic acids	27
Fig.1.3. Diazotization Coupling Reaction (Griess reaction).....	36
Fig.1.4. Molecular structure of nitrates and nitrites molecules.....	42
Fig.1.5. Regulation of blood pressure <i>via</i> sodium retention by the renin-angiotensin system	47
Fig.2.1. Mean <i>Clostridium perfringens</i> populations (log CFU/g) in ham containing various concentrations of NaNO ₂ and sodium erythorbate prepared and processed within 3 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 15 h (Chill)	106
Fig.2.2. Mean <i>Clostridium perfringens</i> populations (log CFU/g) in ham containing various concentrations of NaNO ₂ and sodium erythorbate prepared and processed within 24 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 15 h (Chill)	107
Fig.2.3. Mean residual nitrite levels (mg/kg) expressed as the total curing ingredient in ham (prepared and processed within 3 and 24 h) before heat treatment (cooking at 75°C for 20 min followed by cooling exponentially from 54.4°C to 4°C in 15 h)	108
Fig.2.4. Mean residual nitrite levels (mg/kg) expressed as the total curing ingredient in ham (prepared and processed within 24 h) before and after heat treatment (cooking at 75°C for 20 min followed by cooling exponentially from 54.4°C to 4°C in 15 h).....	109
Fig.3.1. Mean <i>Clostridium perfringens</i> populations (log CFU/g) in ham containing various concentrations of NaCl, NaNO ₂ and <i>Opti.Form</i> PD4®, prepared and processed within 3 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 9 h (Chill).....	130

- Fig.3.2. Mean *Clostridium perfringens* populations (log CFU/g) in ham containing various concentrations of NaCl, NaNO₂ and *Opti.Form* PD4®, prepared and processed within 3 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 15 h (Chill)..... 131
- Fig.3.3. Mean *Clostridium perfringens* populations (log CFU/g) in ham containing various concentrations of NaCl, NaNO₂ and *Opti.Form* PD4®, prepared and processed within 3 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 21 h (Chill)..... 132
- Fig.3.4. Mean *Clostridium perfringens* populations (log CFU/g) in ham containing various concentrations of NaCl, NaNO₂ and *Opti.Form* PD4®, prepared and processed within 24 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 15 h (Chill)..... 133
- Fig.3.5. Mean *Clostridium perfringens* populations (log CFU/g) in ham containing various concentrations of NaCl, NaNO₂ and *Opti.Form* PD4®, prepared and processed within 24 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 21 h (Chill)..... 134

LIST OF TABLES

Table 1.1. Toxin typing system used to classify <i>C. perfringens</i> isolates	5
Table 1.2. United States Centers for Disease Control and Prevention estimates of the most common foodborne pathogens contributing to domestically acquired illnesses in the United States, 2011	11
Table 1.3. United States and European Union regulations regarding the use of nitrites in meat products	25
Table 1.4. Effect of storage time and pH values on residual in meat after heating	31
Table 1.5. Different chromatographic methods principles.....	41
Table 1.6. Countries with the highest salt production rates in the world	44
Table 1.7. Salt and sodium content (per 100 g) in typical meat products.....	51
Table 1.8. Influence of solute on minimal inhibitory A_w values.....	56
Table 1.9. Typical bacterial species associated with foods containing different salt levels	56
Table 1.10. General characteristics of some typical organic acids in foods	59
Table 2.1. Mean pH (\pm SD) and water activity values of ham containing different concentrations of sodium nitrite (from 0 to 200 ppm) and sodium erythorbate (0 and 557 ppm).....	104
Table 3.1. Mean pH (\pm SD) and water activity values of ham containing various concentrations of sodium chloride, sodium nitrite and <i>Opti.Form</i> PD4®	128

INTRODUCTION

Foodborne illnesses is a major cause of illness and death in the United States and more than 9 million cases of foodborne illness are estimated annually in the U.S. (Scallan et al., 2011). Bacterial pathogens are responsible for more than 50% of those cases, with *Clostridium perfringens* being the third most common etiological agent. The main vehicles for *C. perfringens* foodborne illness are foods containing meat and meat products that are temperature abused. *C. perfringens* spores can survive the typical thermal processing protocols used in meat processing and other antimicrobial strategies adopted by meat processors to inhibit bacterial growth in meat products including sodium chloride (NaCl), sodium nitrite (NaNO₂), acidic conditions, low water activity (Aw) values and antimicrobial compounds (Li and McClane, 2006). Changes in product formulation or processing conditions can alter the behavior of the microorganism hence, the risk of survival of the foodborne pathogen during processing, storage and distribution. Consumer interest in organic and healthy food products is increasing in the United States (Jackson et al., 2011a). The market for organic products has expanded significantly and consumers spend more than \$7 billion annually on products processed with minimal amounts of preservatives and chemicals in the past ten years. New meat products are being formulated to fulfill the consumer demand and ingredients such as NaNO₂ or NaCl are being reduced in the standard formulations of cured products. However, NaNO₂ and NaCl are essential ingredients in meat products as they improve the quality (color, texture and flavor) and safety of meat products. Antimicrobial properties of curing ingredients have been recognized previously (Honikel, 2008; Taormina, 2010) and reduction of their concentration in cured meat products is associated with an increased risk of *C. botulinum* and *C. perfringens* foodborne illness. Microbial stability of new meat product

formulations should be evaluated to establish guidelines for safe processing and use by the consumers. Approaches to diminish the impact of new meat formulations on microbial safety include the use of compatible antimicrobial ingredients such as organic acid salts. These products can be used at lower concentrations and contribute to microbial safety of the food products (Theron and Rykers, 2011). The current research evaluated the effect of common ingredients used in the manufacture of cured products (NaNO_2 , NaCl and sodium erythorbate) and organic acid salts on the germination and outgrowth of *C. perfringens* spores in ham. Results and conclusions from this research can assist meat processors to formulate ham with reduced NaNO_2 and NaCl , while assuring the microbial safety of the product.

CHAPTER 1

LITERATURE REVIEW

1. *Clostridium perfringens*

Taxonomy of microorganism

Domain: *Bacteria*

Phylum: *Firmicutes*

Class: *Clostridia*

Order: *Clostridiales*

Family: *Clostridiaceae*

Genus: *Clostridium*

Species: *Clostridium perfringens*

(Bergey's Manual, 2005)

The genus *Clostridium* is comprised of a diverse group of microorganisms including pathogens, spoilage organisms and environmental species. Apart of *Clostridium perfringens*, other pathogens in the genus include *Clostridium botulinum* and *Clostridium tetani*; some species are used commercially for hydrogen production (Lin et al., 2007).

There are more than 100 species in the genus *Clostridium* and *C. perfringens* is more closely related to *C. pasteurianum* based on molecular analysis (Labbe, 2000).

Heterogeneity exists among the *C. perfringens* strains but in general they can be grouped inside a very closely related cluster (Montville and Mathews, 2008). Currently, *C.*

perfringens is classified based on toxin production as important phenotypic and genotypic features are related depending upon the toxin type produced.

1.1 General characteristics

Clostridium perfringens is a Gram positive, large, non-motile rod with square ends. It is an encapsulated microorganism with capacity to form spores, highly resistant to environmental conditions (Ryan and Ray, 2010). As other members of the genus, *C. perfringens* is an obligate anaerobe; however, it can tolerate moderate exposure to air through its capacity to create more reduced conditions in the surrounding environment (Montville and Mathews, 2008). This organism can tolerate and grow at temperatures between 10 and 54°C, with an optimum growth at 42-43°C. The generation time is usually below 12 min under optimum conditions, making the growth of *C. perfringens* considerably faster compared with other microorganisms (Montville and Mathews, 2008). *C. perfringens* is a natural inhabitant of soil, water and the intestinal tract of warm-blooded animals and humans (Sigrid and Granum, 2002). Like in the case of other spore formers, *C. perfringens* has been isolated from almost any environmental sample tested and has been widely recognized as a common foodborne pathogen.

C. perfringens is referred as the most widely distributed pathogen in nature (Lindstrom et al., 2011) and has been implicated as the etiological agent of two important foodborne diseases; the relatively mild, classic Type A diarrhea and the more serious Type C human necrotic enteritis (Sigrid and Granum, 2002). Type A diarrhea is a common syndrome in developed countries whereas human necrotic enteritis is very rare and few outbreaks of this illness have been reported. Recently, some *C. perfringens* strains (especially Type A strains) are identified as the main cause of some cases of antibiotic associated diarrhea

(AAD) and sudden infant death syndrome (SIDS), cases typically attributed to other pathogens like *Clostridium difficile*. Additionally, both AAD and SIDS have been considered traditionally as non-food-related illnesses but some evidence suggests that these syndromes could be related to consumption of food contaminated with *C. perfringens* (Lindstrom et al., 2011). However, this has been confirmed just for strains that carry the *cpe* gene (gene responsible to produce the enterotoxin of *C. perfringens*) in the plasmid.

1.2 Toxin production by *C. perfringens*

The capacity to produce potent exotoxins is the basis to understand the pathogenesis of foodborne illness caused by *C. perfringens*. Fifteen toxins that cause a diverse range of illnesses in humans and animals have been identified for this organism (Lindstrom et al., 2011). One of the most important features regarding these toxins is that they are used to classify *C. perfringens* according to a toxin-typing system. According to this system, *C. perfringens* can be classified in 5 major types (A through E) depending on each isolate's ability to produce one or more exotoxins (alpha, beta, epsilon and iota) (Montville and Mathews, 2008). The *C. perfringens* enterotoxin or CPE is the protein involved in the classic diarrhea affecting humans and all 5 types can produce the toxin (Table 1).

The toxin typing system is used to identify and characterize pathogenic *C. perfringens* strains but it is estimated that less than 5% of all isolates studied have the capacity to produce CPE. From the 5 major types, Type A is the most important and common in human illness; this type of *C. perfringens* is commonly found in soil and the intestinal environment of humans and food animals (Ryan and Ray, 2010). For Type A isolates, the *cpe* gene is located either on the chromosome or the plasmid. Even though it is the same

gene and the same toxin produced, most of the cases of foodborne outbreaks for *C. perfringens* implicate Type A isolates carrying the *cpe* gene in the chromosome (Sigrid and Granum, 2002). The main reasons for this epidemiological finding are the greater prevalence of Type A isolates in the environment and also a recently discovered high resistance of *cpe* chromosomal Type A strains to environmental and stress conditions (Li and McClane, 2006). The chromosomal *cpe* positive strains seem to be more resistant to high levels of NaCl, high and low temperatures and nitrites, hurdles used in food processing to control the outgrowth of pathogenic microorganisms. The final result could be a selective pressure that is promoting a greater prevalence of these types of isolates in the food chain. Additionally, some of these Type A isolates are implicated in myonecrosis, bovine and ovine enteritis and porcine necrotic enterocolitis (Songer and Post, 2005).

Table 1.1. Toxing typing system used to classify *C. perfringens* isolates.

Type	α -toxin	β -toxin	ϵ -toxin	ι -toxin	Enterotoxin
A	+	—	—	—	+
B	+	+	+	—	+
C	+	+	—	—	+
D	+	—	+	—	+
E	+	—	—	+	+

Adapted from Sigrid and Granum. (2002)

1.3 Occurrence of disease and pathogenesis

C. perfringens outbreaks most commonly involve foods rich in meat (Ryan and Ray, 2010) as the organism is not capable of producing over 13 of the 20 amino acids considered essential (Andersson et al., 1995; Ando et al., 1985; Labbe and Huang, 1995). The protein content of meat provides *C. perfringens* with all the components necessary for germination and growth. As it can be expected, other protein rich foods can be related

with some outbreaks as is the case of gravies, stews and Mexican food (Montville and Mathews, 2008). *C. perfringens* can be isolated from vegetables as well but outbreaks involving fresh produce are uncommon; it is most likely that vegetables serve as a source for the contamination of meat containing foods.

Once present in meat products, the spore formation capacity and the rapid growth of *C. perfringens*, represents a special hazard in foods that are temperature abused. If *C. perfringens* spores are present, high temperatures during cooking will promote germination and outgrowth as this condition is interpreted by the microorganism's physiological signal that other competitors were eliminated (Ryan and Ray, 2010). Naturally, spores will survive the normal cooking procedure because the D value for those in broth is estimated to be as high as 100 min at 100°C for some strains (Sarker et al., 2000). Germination may be accompanied with outgrowth for a sufficient time period to reach a minimum population of about 10^{6-7} organisms/g of food that is considered the infectious dose for *C. perfringens* (Montville and Mathews, 2008). In comparison with other foodborne pathogens, this infectious dose is high and is explained in terms of the low survival of vegetative cells in the digestive tract of humans.

Temperature abuse is a key factor that will provide the conditions for *C. perfringens* outgrowth and this could happen in two ways; slow cooling of cooked products (Eg. cooling of heated food at room temperature) or inadequate reheating that will allow vegetative cells to multiply or the remaining cells to sporulate (McClure, 2002). Ingestion of foods containing high populations of vegetative cells is the main infection route for humans; the bacteria will colonize the small intestine affecting the ileum section. After a first exposure to acidity in the stomach and later to high alkaline conditions in the

intestine, the vegetative cells sporulate, releasing high amounts of CPE during lysis of the mother cells (Montville and Mathews, 2008).

In the small intestine, CPE attaches to the epithelial cells through the recognition of a brush border receptor on the surface of epithelial cells (Fig. 1). The mechanism is explained in terms of the formation of a small protein complex that will act as a pore, promoting the Ca^{2+} dependent release of low molecular weight metabolites and ions (Ryan and Ray, 2010). The final result is the occurrence of acute diarrhea as a consequence of electrolyte imbalance in the intestine. The whole process takes about 8 to 24 h. Most of the cases are self-limiting as the intestinal cells affected by the toxin die as a result of increased Ca^{2+} levels (Lindstrom et al., 2011). The typical symptoms include watery diarrhea, abdominal cramps and vomiting in few cases; fever is not a common feature.

Most of the outbreaks involve large number of cases but the majority is mild situations where recovery occurs after 24 to 48 h. Fatalities may occur in debilitated patients, infants or elderly people. Presence of the preformed CPE toxin is not common as food does not provide conditions for sporulation. CPE is heat labile and is destroyed with mild cooking to 60°C during 10 min (Montville and Mathews, 2008).

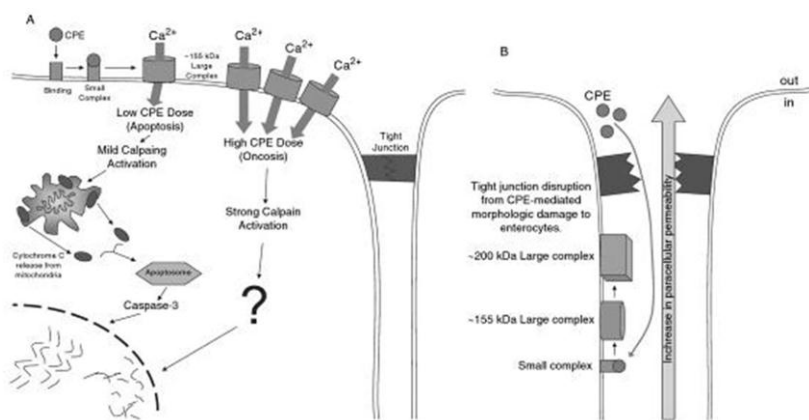


Fig.1.1 Pathogenesis of Type A foodborne illness by *C. perfringens* enterotoxin (CPE) (Alouf and Popoff, 2006).

1.4 Historic background and current health impact

The American scientist William Henry Welch discovered *Clostridium perfringens* in 1891 during an autopsy of a person who died by severe hemorrhages from an ulcer. At the beginning, the observed organism was named as *Bacillus aerogenes capsulatus*, but was later changed to *Clostridium welchii* (Ledermann, 2007). This species was called *C. welchii* until the 1970's (Labbe, 2000) and since the beginning was recognized mainly as the etiologic agent of gas gangrene; it is estimated that gas gangrene caused by *C. perfringens* accounted for many deaths in the American army during World War I (Shimizu et al., 2001). Early in 1899, the first report of *C. perfringens* associated diarrhea was mentioned in the literature associated with rice pudding as the suspected vehicle (ICMSF, 1996). However, strong evidence about foodborne illness associated with this organism was mentioned for the first time during the 1940's and 1950's. Knox and McDonald in 1943 identified an outbreak in a school setting where several children became ill after eating gravy contaminated with *C. perfringens* (Labbe and Juneja, 2006). Later, in 1945, another four outbreaks were reported, this time involving cooked chicken

that was previously steamed (Labbe and Juneja, 2006). As it is observed, since the first outbreak reports meat products or protein-rich foods were implicated as the main vehicle for *C. perfringens* transmission. The final confirmation about the role of *C. perfringens* as a food borne pathogen was concluded after the pioneering work of Hobbs and colleagues in Great Britain (Montville and Mathews, 2008) where some of the main characteristics associated with this bacterium were established, e.g., spore formation capacity and mild heat resistance. After the discovery and isolation of the enterotoxin during the 1970's a better understanding about the pathogenesis of Type A diarrhea was achieved and now *C. perfringens* is recognized as one of the most common causes of food borne illness in the world.

Some of the most famous *C. perfringens* outbreaks are the ones that occurred during Saint Patrick's Day in Cleveland, OH and Virginia in 1993, after serving an improperly reheated corned beef causing 156 and 86 sick people, respectively (Montville and Mathews, 2008). Since then, several more outbreaks have been reported. According to CDC data, from 1993 to 1997, there were 40 *C. perfringens* outbreaks reported which accounted for more than 41% of the total foodborne disease outbreaks during the same period, resulting in a total of 2,772 cases. Also, the CDC reported 6,742 new cases with four fatalities in the period from 1998 to 2002. Other countries have reported an important number of cases of foodborne illness associated with *C. perfringens* as well. For example, authorities in Norway estimate that more than 30% of all the cases of food-related illnesses are attributed to this pathogen (Lindstrom et al., 2011). Currently, the CDC estimates that more than 250,000 cases with at least 7 deaths occur every year in the United States, taking into account that most of the cases are not reported to health

authorities. The economic cost associated with these cases is estimated to be more than \$240 million.

Scallan et al. (2011) estimated that *C. perfringens* ranked third among all the pathogens reported, accounting for 10% of all the confirmed cases; *C. perfringens* was just behind norovirus and non-typhoidal *Salmonella* spp. in the total number of illnesses (Table 2).

The authors reported that around 900,000 cases domestically acquired may occur annually but only around 2,000 of these episodes are confirmed through laboratory diagnosis. While the number of hospitalizations and deaths due to *C. perfringens* illness are low, these estimates may change as the population of elderly and immunocompromised persons tends to increase in developed countries.

1.5 Implicated foods and control

The intestinal tract of food animals is the principal source of contamination and both spores and vegetative cells may enter the food production chain during slaughter (Montville and Mathews, 2008). Unfortunately there is no concordance between data obtained with molecular and culture procedures where the incidence reported from PCR analysis is much higher than with traditional microbiological procedures (Lindstrom et al., 2011). Some data suggests that humans may be the main reservoir and the ultimate source of contamination as the organism has been isolated from the intestinal tract of healthy humans (Carman et al., 2008). The prevalence of *C. perfringens* in foods of animal origin is calculated to be ca. 12% according to molecular data, but few isolates have been recovered from these samples (Lindstrom et al., 2011).

Table 1.2. United States Centers for Disease Control and Prevention estimates of the most common foodborne pathogens contributing to domestically acquired illnesses in the United States, 2011.

Pathogen	Estimated number of illnesses	90% Credible interval	%
Norovirus	5,461,731	3,227,078–8,309,480	58
<i>Salmonella</i> spp.	1,027,561	644,786–1,679,667	11
<i>C. perfringens</i>	965,958	192,316–2,483,309	10
<i>Campylobacter</i> spp.	845,024	337,031–1,611,083	9
<i>Staphylococcus aureus</i>	241,148	72,341–529,417	3

Scallan et al. (2011)

Several reports about the prevalence of this pathogen in different meat and poultry products are available in the literature. For example Miwa et al. (1998) reported in Japan a prevalence of *C. perfringens* of 16% and 10% for raw beef and raw pork, respectively. *C. perfringens* prevalence as high as 84% in raw chicken was reported using molecular analysis. Stagnitta et al. (2002) reported a prevalence of 26% for sausages and 19% for hamburgers in Argentina. In the case of cured products another study determined a prevalence of 1.6% of vegetative cells for whole muscle cured meat whereas a prevalence as high as 48.7% in cured ground or emulsified samples was also reported (Taormina et al., 2003). Cured products can also support growth of *C. perfringens* such as ham, cured sausages and hot dogs (Crouch and Golden, 2005). *C. perfringens* activated spores were capable to grow in cured frankfurters when incubated at 37°C and 23°C (Solberg and Elking, 1970).

Use of chemical preservatives such as organic acid salts (lactates, acetates, citrates) and sodium nitrite represent means to control the germination and outgrowth of *C. perfringens* spores. Food product's intrinsic factors like A_w , pH and the use of other preservatives could greatly minimize the risk of germination and outgrowth of *C.*

perfringens. A minimum concentration of 2.5% of NaCl and 0.4% of sodium nitrite is considered necessary to inhibit the growth of vegetative cells containing a chromosomal *cpe* gene by 1 log (Li and McClane 2006). However, most of the meat products currently in the market contain NaCl and sodium nitrite concentrations that are much lower than the ones used in some of the investigations. The USDA-FSIS approved the use of citrates and lactates for meat and poultry products (USDA-FSIS, 2000). Ingredients like sodium and potassium salts of organic acids, including citric, acetic and propionic acid can minimize the risk of *C. perfringens* germination and outgrowth (USDA-FSIS, 2000). As sodium chloride and sodium nitrite may not be inhibitory enough to control *C. perfringens*, the use of organic acids salts represents an attractive alternative for meat industry.

It is clear that the inclusion of antimicrobial compounds is relevant in the cases where cooling deviations are present and the product is subjected to abusive storage temperatures. Proper cooking and cooling is the main approach for controlling *C. perfringens* germination and outgrowth. *C. perfringens* spores are heat resistant and they can germinate after exposure to mild cooking temperatures; germinated spores can multiply rapidly in food if this is not cooled in a short time (Thippareddi et al., 2003). To mitigate the risk of *C. perfringens*, the USDA-FSIS established performance standards for stabilization (cooling) of meat and poultry products. These guidelines recommend that cooked meat and poultry products should be cooled from 54.4°C (130°F) to 26.6°C (80°F) within 1.5 h and from 26.6°C to 4.4°C (40°F) within 5 h. Cured products should be cooled from 26.6°C to 4.4°C in 10 h (USDA-FSIS, 1999). The main purpose is to reduce the time the product stays at the optimum temperature range for *C. perfringens* growth (42-43°C).

2. Sodium nitrite for curing and preservation of meat products

2.1 General information

Sodium nitrite (NaNO_2) is used as a preservative and curing agent in meat products (Benjamin and Collins, 2003). This compound is produced from nitrogen oxides or nitrous fumes that are obtained after the catalytic oxidation of ammonia with air in a controlled environment. The nitrogen oxides are then absorbed in a solution of sodium carbonate or sodium hydroxide to obtain pure, white to yellowish color (Hayes and Britton, 1936). The chemical reaction to illustrate this process is shown below:



Sodium nitrite is sold as a mixture of sodium chloride (93.75%) and sodium nitrite (6.25%) commonly termed in the meat industry as the “Prague powder # 1, “Curing salt” or “Pink salt”. As sodium nitrite concentrations in cured products is very low (ppm), it is mixed with sodium chloride to facilitate the ingredient’s manipulation. “Prague powder # 2 contains a mixture of sodium chloride (89.75 %), sodium nitrate (4 %) and sodium nitrite (6.25 %) that is used for fermented products (Sarraga et al., 1989). Curing salts are commonly colored with a red dye (FD & C Red #3, approved by the Federal Food, Drug and Cosmetic Act) to produce a pink color to the product and to prevent confusion between the curing salts and NaCl.

Nitrogen present in nitrite is usually in the oxidative state 3+, meaning that it can be oxidized or reduced; it means that nitrite is considered a very reactive compound. When exposed to normal environmental conditions, nitrite is oxidized to nitrate (NO_3). Under acidic conditions, nitrite can be physiologically reduced to nitric oxide (NO), by

microbial reductases (Zumft, 1997). However, the ultimate source for nitrite in nature is the reduction of nitrate, a compound of low chemical reactivity that is ubiquitous in soil, water and plant materials as a consequence of the nitrogen cycle (Benjamin and Collins, 2003). Currently, extensive use of fertilizers has become an important source of nitrates in the environment (Roberts, 1996). As a consequence, fruits and vegetables account for almost 80% of the total nitrate consumed in the diet (Hord et al., 2009). According to the National Research Council Committee on Nitrite and Alternative Curing Agents in Food (NRC), the daily intake on nitrate in the United States is approx. 73 mg/person and most of this is consumed through vegetables (Santamaria, 2006). On the other hand, Knight et al. (1987) estimated a daily nitrite and nitrate intake of 1.4 and 95mg/person respectively, with cured meats as the main source of nitrite.

2.2 Historic background of meat curing

Curing is the treatment applied over food or other products, like tobacco, to prevent decay by the addition of salt or smoke (Cambridge University Press, 2011). The curing process refers to food preservation by drying or removal of water. It is difficult to track the exact origin of the curing practice in human civilization, as it has been present in history for thousands of years (Roberts, 1996). The main purpose of curing was to preserve the food in times where refrigeration was not available, and was especially important for meat products.

Traditionally, salt was rubbed onto the surface of the meat and in some cases it was observed that this process produced a change in the color. The formation of a pink color in cured meats became a desirable characteristic since the time of Homer (850 B.C.), and

the curing practice was later adopted by the Romans who learned many of the ancient Greek traditions (Kramlich et al., 1973). The traditional curing process was possible by the unknown presence of nitrate as a contaminant in common salt; nitrate in salt was later reduced to nitrite by bacterial metabolism making the pink color possible. It was until the late 19th century that the presence of “Saltpetre” (KNO_3) was formally recognized (Honikel, 2008). A complete understanding of the role of nitrates and nitrites in the curing process was elucidated in the early 20th century.

Polenske, a German scientist, provided technical evidence that “Saltpetre” can be converted to nitrite by the action of microorganisms in an experimental solution (Pegg and Shahidi, 2000). Additionally, Lehman and Kisskalk demonstrated in 1899 that nitrite was the chemical compound responsible for the pink color and other characteristics in cured meat products. Haldane in 1901 investigated more closely the red pigment present in cured products; he prepared nitrosylhemoglobin (NOHb) by adding nitrite to hemoglobin and showed that its conversion to nitrosylhemochromogen after thermal processing was the pigment responsible for pink color (Pegg and Shahidi, 2000; Honikel, 2008). The final explanation was given by Hoagland in 1914 who reported nitrous acid (HNO_2) or a metabolite similar to nitric oxide (NO) as the reactant in the chemical reaction leading to curing. These scientific discoveries led to a formal introduction of the current curing process, where nitrite is included as part of the formulation of cured products. Use of nitrites is an advantage as the addition of nitrate yielded extremely variable amounts of nitrite in the final product. The USDA approved the direct use of sodium nitrite for curing purposes in all the federal inspected establishments in 1926 (Pegg and Shahidi, 2000).

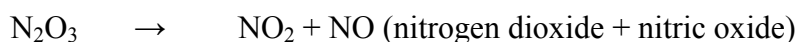
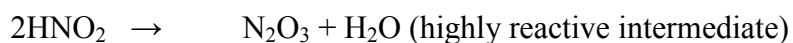
2.3 The chemical process of meat curing

The pink color of cured products is a product of the reaction between nitrite and compounds present in the meat. Currently, sodium nitrite is used to enhance the color of many products including ham, bacon, bologna and hot dogs (Montville and Mathews, 2008). Meat color is one of the main criteria consumers like to judge for product quality; the other aspects are tenderness, juiciness and flavor that can be affected by nitrites (Lueck, 1980). Nitrites contribute to meat flavor and texture functioning as antioxidant compounds (Benjamin and Collins, 2003). One of the important functions of nitrites in meat systems is their role as antimicrobial agents, especially, their control of the germination and outgrowth of *Clostridium botulinum* and other sporeforming bacteria like *C. perfringens*. Antimicrobial activity of nitrites is also explained by chemical reactions with meat components but some of the mechanisms have not been yet elucidated (Pegg and Shahidi, 2000).

The curing process will start with the conversion of nitrate to nitrite by the action of bacterial reductases from the native microflora of the meat such as in fermented cured products, whereas in other non-fermented products nitrites are added directly (Tompkin, 2005). Nitrites undergo a series of reactions involving H^+ ions, reductants, meat pigments, salt, proteins, radicals, bacterial components, lipids and flavor precursors (Cassens et al., 1981). The most important reactions during curing are those involving meat pigments, particularly myoglobin. Myoglobin is the native pigment that contributes to meat color; it is present at different concentrations depending on the animal species or the type of muscle used. Myoglobin is a single polypeptide of 153 amino acid residues with one molecule of heme (Osgood and Occor, 2009). Myoglobin has been found as part of the

muscle tissue of all mammals and functions mostly as an oxygen reservoir (Osgood and Occor, 2009). Myoglobin facilitates oxygen diffusion in the tissues in situations where the muscle is working hard and more anaerobic conditions prevail (Seifter et al., 2005).

The oxidation state of the iron present in the heme group of myoglobin determines the color of the meat pigment (Osgood and Occor, 2009). In the normal physiological state, iron appears as Fe^{+2} , giving the meat a reddish-purple color. After slaughter, oxygen attaches to myoglobin to produce oxymyoglobin that has a cherry-red color typical of fresh meat. Myoglobin and oxymyoglobin are also oxidized to form metmyoglobin where the iron is in the oxidative state +3 and the meat presents a brown color. Inhibition of reducing enzyme activity and vacuum packaging promote the appearance of metmyoglobin (Pegg and Shahidi, 2000). Nitric oxide is one of the products of the reduction process undergone by nitrites. This compound interacts with myoglobin to establish the cured color. During nitrite reduction other intermediate reactive species are produced (Benjamin and Collins, 2003):



This process is accelerated by the presence of acidic conditions, bacterial reductases, natural reductants (NAD), cysteine, cytochromes and water. Most of the added nitrites (80 %) are reduced to nitric oxide (Honikel, 2008). Artificial reductants like ascorbate and isoascorbate or erythorbate are added as part of the formulation to accelerate the formation of nitric oxide (NO) (Benjamin and Collins, 2003):



Nitric oxide reacts with metmyoglobin to form nitrosylmetmyoglobin. In the presence of reductants (naturally present or erythorbate), nitrosylmetmyoglobin is transformed into nitrosylmyoglobin, with the iron again in the oxidative state +2. This compound is not stable enough to produce the cured pink color but it can be degraded with heat to generate nitrosylmyochrome, the final cured pigment. If nitrosylmyochrome is exposed to light or air, iron is reoxidized to the ferric form giving the meat a brown color.



Some studies reported that the concentration of sodium chloride can also affect the rate of how nitrite is converted to nitric oxide (Sebranek and Fox, 1985). This process is also accelerated in the presence of ascorbates and erythorbate. Other reactions related to the curing process involve meat proteins with the subsequent formation of disulfide bonds and more nitric oxide. This reaction has importance in terms of meat texture quality by the crosslinking of proteins through disulfide bonds (Pegg and Shahidi, 2000). More reactions between nitrites and proteins contribute to the formation of carcinogenic compounds (nitrosamines) not related to the normal curing process.



2.4 Antimicrobial aspects of nitrites in cured products

Nitrite is an antimicrobial compound with a bacteriostatic effect against a wide range of microorganisms. Conditions present in the meat system that influence the antimicrobial properties of nitrites include pH, redox potential (Eh), chemical composition of the sample (including salt, phosphates or other antimicrobials) and the temperature of storage (Roberts, 1996). Steinke and Foster demonstrated for the first time the antimicrobial action of nitrites against *C. botulinum* and other putrefactive microorganisms (Benjamin

and Collins, 2003). Since then, nitrites have been used as an approach to prevent the occurrence of botulism from meat products. However, nitrites are also active against other sporeforming pathogens such as *C. perfringens* and *Bacillus cereus*, and also against many spoilage microorganisms. Nitrite can control sporeformers by inhibiting the outgrowth of germinated spores at the concentrations usually present in meat products (100-200 ppm). Spore viability is affected at much higher nitrite concentrations than the normal amounts used in meat products (Li and McClane, 2006).

The antimicrobial properties of nitrite have been demonstrated and studied for aerobic and anaerobic microorganisms. In the case of aerobic bacteria the main site of antimicrobial activity of nitrite is the cell membrane and all the metabolic processes associated with this structure (Roberts, 1996). However, the precise site of antimicrobial action is dependent on the bacterial species. For *Escherichia coli* and *Pseudomonas aeruginosa* the main site of inhibition is the enzyme aldolase (Yarbrough et al., 1980). In the case of *S. aureus*, strong evidence suggests that inhibition occurs in the sulphhydryl groups of the coenzyme A or at lipoic acid cofactors involved in pyruvate metabolism (Buchanan and Solberg, 1972). Inhibition of the enzyme pyruvate decarboxylase was demonstrated for brewer's yeasts (McMindes and Siedler, 1988). Antimicrobial nitrite effect has been also demonstrated against *Achromobacter* spp., *Enterobacter* spp., *Flavobacterium* spp., *Micrococcus* spp., *Pseudomonas* spp. and *Listeria monocytogenes* (Montville and Mathews, 2008).

The inhibition of pyruvate metabolism observed in aerobic microorganisms is related to the inhibitory effect observed for anaerobic species. McMindes and Siedler (1988) demonstrated the inhibition of the pyruvate:ferredoxin oxidoreductase system in *C.*

perfringens with both nitrite and nitric oxide. Reddy et al. (1983) and Carpenter et al. (1987) determined that NO is more inhibitory than nitrite against anaerobic microorganisms. The role of NO consists in the reaction with non-heme iron centers of many enzymes such as ferredoxin and hydrogenase. The affected enzymes are part of some metabolic steps during glycolysis via the Embden-Meyerhof pathway and the pyruvate metabolism, an important source of energy for the cell. Other indications of the antimicrobial mechanism of nitrite have been elucidated from human physiology studies. Dietary nitrite plays a major role in defense mechanisms in the stomach against enteric pathogens, through a process dependent on oxidative reactions under acidic conditions that leads to the formation of toxic peroxynitrite (Dykhuizen et al., 1996).

Inhibition of microbial growth by nitrites depends on the organoleptic conditions of the meat system evaluated as well as the type of microorganism involved. A concentration above 150 ppm is considered appropriate to prevent the occurrence of *C. botulinum* spores germination and outgrowth and food spoilage by putrefactive microorganisms (Roberts, 1996). The relation between nitrite concentrations and microbial inhibition under specific conditions has been studied in terms of residual nitrite. Christiansen et al. (1973) concluded that input nitrite was more important than residual nitrite to predict the safety of canned cured ham using different nitrite levels from 0 to 500 ppm. Christiansen reassessed the influence of residual nitrite levels on antibotulinal action. In cured products stored for long periods of time lower residual nitrite levels were related with a higher risk of botulism in cured products that are temperature abused (Christiansen et al., 1978). Tompkin et al. (1979) commented that residual nitrite levels are essential for proper antibotulinal effect and high levels of reducing agents (isoascorbate) may decrease the

inhibitory power attributed to these compounds; the same author suggested that residual nitrite may interact with iron in metabolic enzymes that are essential for microbial survival. Reduction in residual nitrite can also result in a higher dissociation of the toxic nitric oxide from iron in the meat system (Tompkin, 2005). Depletion in residual nitrite levels over the storage of sausages increases microbial spoilage by aerobic microorganisms (Bozkurt and Erkmen, 2004). Higher levels of residual nitrites are correlated with lower concentrations of *L. monocytogenes* in cured products that are vacuum packed and stored at 5°C (Grau and Vanderlinde, 1992). Also, it has been demonstrated that a minimum level of 3µM of residual nitrite is necessary to double the time for more than 3log of growth of *L. monocytogenes* in cured products (Duffy et al., 1994). Other compounds like sorbic acid plays a role in nitrite inhibitory activity against *C. botulinum*. Sofos et al. (1980) reported that higher *C. botulinum* inhibition was observed when residual nitrite concentrations remained high at the presence of sorbic acid.

2.5 *C. perfringens* sensitivity to nitrites

Labbe and Duncan (1970) speculated about the toxicity of sodium nitrite against *C. perfringens* after the observation that heated sodium nitrite in culture media was more toxic for this pathogen than the non-heated one. Sodium nitrite inhibits the outgrowth of germinated spores and can induce germination of dormant spores making them more susceptible to intense heat treatments. Moran et al. (1975) identified that the toxic compounds generated from heated nitrite are implicated in reactions involving ferrous sulfate and ammonium disulfide. These interactions were confirmed by O'Leary and Solberg (1976) after the observation of enzymatic inhibition due to chemical reactions of

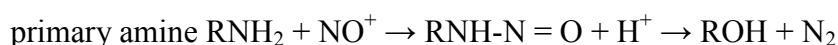
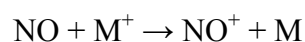
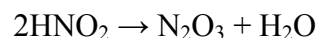
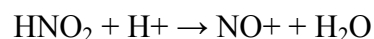
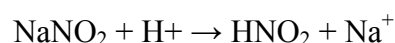
nitrite and thiol groups within the cell, confirming the role of S-H groups for the antimicrobial effect of nitrite against *C. perfringens* and other bacteria. Currently, it is not clear if these antimicrobial mechanisms are active inside the meat systems. However, some experiments have established that in broth systems the minimal inhibitory concentration of nitrite for *C. perfringens* could be as high as 12,000 ppm (Gough and Alford, 1965). In meat systems, the sensitivity of *C. perfringens* is increased by the presence of other curing salts (NaCl, phosphates) or antimicrobial compounds present in the formulations. A minimum concentration of 200 ppm of sodium nitrite is considered enough to inhibit *C. perfringens* spore germination and outgrowth; this has been demonstrated for products such as frankfurters (Jackson et al., 2011a) and Mortadela type-sausages (de Oliveira et al., 2011). The genetic background of the strains contaminating the product also influences the nitrite sensitivity of *C. perfringens*. *C. perfringens* strains that carry the *cpe* gene as part of the chromosome are as twice as resistant to sodium nitrite than other strains carrying this gene on a plasmid (Li and McClane, 2006). Most of the strains implicated in outbreaks carry the *cpe* gene on the chromosome.

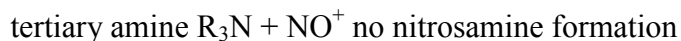
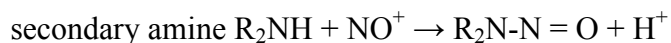
As *C. perfringens* is sensitive to sodium nitrite at the concentrations commercially used in cured products, few outbreaks involving these products have been reported. From 1978 to 1992 only two outbreaks associated with ham were reported (Crouch and Golden, 2005). Recent trends towards the reduction of sodium content in meat products and the use of natural nitrite sources have motivated new studies to evaluate the risk associated with new formulations in cured products (Jackson et al., 2011a; Jackson et al., 2011b). Predictive models for *C. perfringens* growth under abusive cooling are available in the

scientific literature for different types of cured products including beef (Juneja et al., 2001; Sanchez-Plata, 2004), pork (Amezquita et al., 2005; Juneja et al., 2006), chicken (Juneja and Marks, 2002) and turkey (Sanchez-Plata, 2004).

2.6 Nitrite regulation in meat products and health concerns

Nitrites react with amino acids or amino groups in proteins to produce toxic compounds called N-nitrosamines (Pegg and Shahidi, 2000). Since the 1960's, a great concern arose regarding the carcinogenic potential of nitrosamines, leading to a series of regulatory changes in order to control the use of nitrites in meat products. John Morrison Barnes and Peter McGee made the first link between cancer and nitrosamines after the demonstration of the capacity of these compounds to produce liver cancer in rats (Witschi, 2002). Later, several studies demonstrated the presence of high amounts of nitrosamines in cured products, like bacon, subjected to high temperatures (Fiddler et al., 1978). There are more than 300 different types of nitrosamines and about 97% of them have been shown to be teratogenic in laboratory animals. However, no scientific evidence exists that nitrosamines have been the cause of any type of cancer in humans (Pegg and Shahidi, 2000). The chemical process that leads to the formation of nitrosamines in meat systems is depicted below.





This chemical process involves the same reactions leading to the formation of nitric oxide and nitrous acid. Therefore, the same conditions leading to the reduction of nitrite (like low pH) will favor nitrosamine formation. The reactions involve primary, secondary or tertiary amines, with secondary amines forming the more stable nitrosamines. Amines are present in very low concentrations in fresh meat products in the form of creatine, creatinine or free amino acids like proline or hydroxyproline (Honikel, 2008).

Nitrosamine formation happens in vivo if nitrites and other precursors are acquired from the diet as already demonstrated in human subjects (Fine et al., 1977). The latter means that the concern regarding nitrosamines is related to both the nitrite fraction that already reacted with meat components and the residual nitrite.

Nitrite itself can be toxic if consumed in very high quantities; it can interact chemically with hemoglobin and interrupt the normal oxygen transportation in humans. The lethal nitrite dose for humans is established to be between 30 and 250 mg/kg of body weight (Schudeboom, 1993). Nitrite sources for humans include others than cured meats like polluted air, vegetables and contaminated water. It is estimated that around 70% of the nitrites consumed from diet are taken from vegetables and less than 10% are taken from cured meat products (Chow, 2008). Meat products may contain up to 4.6 mg/kg of nitrite and as high as 400 mg/kg for some vegetables in cases of fresh produce (Keeton et al., 2009). However, an effort is made to control the nitrite fraction present in meat products in order to fulfill the WHO recommendation of an acceptable daily intake of nitrite between 0.0 and 0.07mg/kg of body weight (200 mg on average) (WHO, 2007). In the

United States, the average nitrite daily intake has been established to be between 0 and 20 mg (Hord et al., 2009); this represents a low risk according to WHO recommendations. A study made in New Zealand found that the daily nitrite intake represents around 10% of the acceptable limit, but other factors such as nitrate intake from other sources may increase dramatically the real nitrite intake value (Thomson et al., 2007).

Many countries have developed specific regulations to control the amount of nitrate and nitrite present in meat products. Most of the regulations specify the amount of nitrite that must be added to the product as part of the manufacturing process (Roberts, 1996). To control the amount of nitrite input in most of the products and for the possibility for in vivo nitrosamine formation, some regulations establish limits for both the ingoing and residual nitrites. The European Parliament and Council published a revised directive (2006) regarding the use of nitrates and nitrites in cured products including residual nitrite regulations. In the United States, the Code of Federal Regulations (2005) states the maximum concentration of nitrites in meat products (Table 3).

Table 1.3. United States and European Union regulations regarding the use of nitrites in meat products.

Ingredient	Meat Product	US Regulation	European Regulation
Potassium nitrite	Meat products	200mg/kg	150mg/kg
Sodium nitrite	Most of cured products except sterilized meat products in Europe.	200mg/kg	50-175mg/kg expressed as residual nitrite

(Directive, 2006; Code of Federal Regulations, 2005)

For European regulations, residual nitrite is the basis for monitoring and quality control programs. In the United States, the nitrite regulations specify responsibilities for USDA-FSIS when checking the performance and work of meat industries with special attention

on the amount of nitrite used during processing (Code of Federal Regulations, 2005). For other cured products like bacon, the US regulations specify a maximum nitrite input of 120 mg/kg of product in combination with constant monitoring of samples for nitrosamines. In Europe, the regulation specifies a limit of 175 mg/kg of product as residual nitrite.

The health impact of nitrites is still under debate. New evidence suggests that nitrates and nitrites in foods may not represent a health risk and scientific work has been made to illustrate this concept. It is clear the role of nitrite in the normal function of the cardiovascular system as a precursor of nitric oxide and how important it is for a proper immune response (Osgood and Occor, 2009; Montville and Mathews, 2008). In some cases the dietary nitrite intake is much higher than the WHO recommendation and it seems that this high consumption is not associated with harmful health effects (Hord et al., 2009). In fact, there is a correlation between nitrate consumption and reduction of blood pressure; nitrate is reduced to nitrite by commensal microorganisms present in the mouth and later, by the action of nitric oxide synthase, nitric oxide is generated. NO helps to increase vasodilatation (Katan, 2009). Sodium nitrite therapy helps with the regeneration of necrotic tissue in heart and liver after ischemic events thanks to the same physiologic mechanisms involving nitric oxide (Kumar et al., 2008). This effect also promotes normal heart and neurological functions after a heart attack (Dezfulian et al., 2009). Nitrate and nitrite reduction in the mouth leads to generation of toxic compounds like nitric oxide, that has antimicrobial activity enhanced by acidic conditions present in the stomach; a series of mechanisms involving reactive oxygen species and nitrites plays a role in the normal defensive barriers in the digestive tract (Dykhuizen et al., 1996).

2.7 Presence of sodium erythorbate in cured products.

Sodium erythorbate (also known as sodium isoascorbate) is the sodium salt of the erythorbic acid which is the stereoisomer of ascorbic acid (Fig. 1.2) (Fennema, 2008). Erythorbate shares many chemical features with ascorbic acid, including the antioxidant properties that are useful for preservation of meat, poultry and soft drinks (Theron and Rykers, 2011). In cured products, the antioxidant capacity of erythorbate is used to promote a higher reduction of nitrite, enhancing the development of cured colors in a faster and more stable rate (Roberts, 1996). Other antioxidants, like ascorbate, are used in the same way for curing purposes.

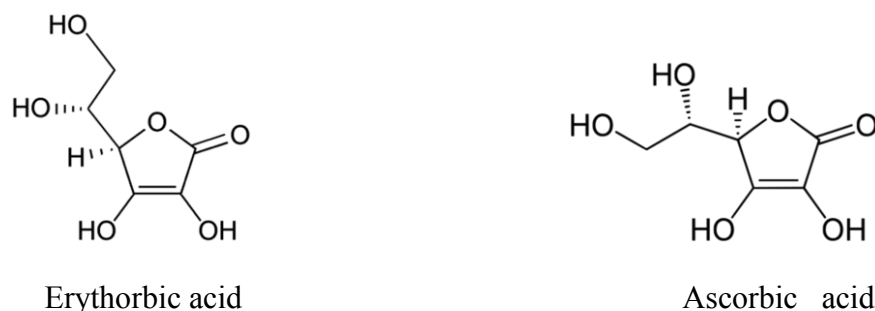


Fig. 1.2 Molecular structures of erythorbic and ascorbic acids

As other antioxidants, sodium erythorbate degrades rapidly in the presence of oxygen, metal ions and light (Osgood and Occor, 2009). Reactive oxygen species (ROS) are common products of chain reactions started by molecular oxygen; these reactions involve the release of electrons that are absorbed by the erythorbate molecule. This mechanism stops those chain reactions that can oxidize other compounds like lipids, proteins or food ingredients like nitrites (Fennema, 2008). Reduced conditions promoted by erythorbate accelerate the formation of nitric oxide from nitrites, especially when acidic conditions

are present in the meat system. By interacting with nitrous acid, sodium erythorbate prevents the formation of nitrosamines in cured meat products, but it also increases the concentration of nitric oxide as part of the curing process (de Man, 1999). The final result is a faster formation of the curing pigment.

Erythorbate and other reductants promote a higher reduction of residual nitrite content. The presence of isoascorbate has a dramatic effect on residual nitrite levels in low pH products (Gibson et al., 1984). In bologna formulated with different erythorbate levels, the residual nitrite decreased faster if the amount of the reductant was increased (Lin et al., 1980). However, the total effect of isoascorbate is better appreciated after several days of storage. Presence of reductants like ascorbates can help to reduce residual nitrite even a 50% after production and heat treatment (Fox and Nicholas, 1974). Also, erythorbate was recognized as a relevant factor for antimicrobial control in meat products (Robinson et al., 1982). Tompkin et al. (1978) demonstrated how the presence of small amounts of sodium isoascorbate enhanced the antibotulinal effect of nitrites, even at the lower concentrations. The mechanism proposed is the capacity of isoascorbate to sequester iron in meat, not making it available for bacterial growth (Tompkin, 2005). This phenomenon has been demonstrated in freshly prepared products. However, isoascorbate also has a detrimental effect on microbial inhibition in cured meats by depletion of residual nitrite (Tompkin et al., 1979).

3 Determination of nitrite in cured meat products

3.1 Presence of nitrite in meat products

Health concerns motivate monitoring of nitrite in cured meat products. In cured meat products, many factors have the potential to influence the concentration of residual nitrite:

the sodium chloride content, amount of added nitrite, chemicals like phosphates or ascorbates, thermal treatments, storage conditions and pH of the product (Gibson et al., 1984). It has been reported that nitrite depletion is more pronounced in the presence of ascorbates and after high thermal treatments; storage at high temperatures promotes higher nitrite reductions and the rate of nitrite loss is not directly related with the amount of added nitrite (Scientific panel on Food Additives, 2003). Even though some regulations regarding nitrite content in meat products are based in residual nitrite measurement, this determination is considered of little value for a real estimation of the initial added nitrite. In terms of food microbiology, there is no clear agreement regarding the usefulness of residual nitrite levels to predict the safety of cured products. In some circumstances, the capacity of a cured product formulation to prevent the growth of pathogens is not related to residual nitrite content (Roberts, 1996).

Several studies determined the influence of different factors in residual nitrite in cured products. Hill et al. (1973) observed that residual nitrite levels were still detectable in frankfurters and sausages after 1 week of storage at 5°C and that this value represented about 25% of the initial added nitrite. Fox and Nicholas (1974) studied the effect of exogenous and endogenous compounds on nitrite losses in cured meats; they reported that ascorbate, cysteine and histidine account for a significant reduction in nitrite in comparison with other substances. Effects of storage temperatures was studied by Wootton et al. (1985); they reported that freezing temperatures account for an important reduction of residual nitrite in cured products like ham, salami and corned beef. In this study, residual nitrite levels were reduced almost half after 24 h of storage at -18°C. The effect of storage time and pH of the product on residual nitrite values is shown in Table 4.

Dordevic et al. (1980) confirmed that higher pH values retard the loss of residual nitrite and a period as long as 60 d is needed to observe a total reduction. On the other hand, unheated products show a shorter decline in nitrite when compared to heat-treated samples. Interestingly, when these unheated samples are analyzed over time the decline in residual nitrite levels is faster than in heated samples, possibly by higher activity of non-degraded compounds in the system (Gibson et al., 1984). The same study showed how the presence of reductants like isoascorbate made the nitrite undetectable after 20 days of storage at 15°C in products with pH values between 5.8 and 6.1. In most cases the nitrite levels are reduced about 50 % after processing and the compound is undetectable after several days of storage (Gry et al., 1983). Some of the factors mentioned here have similar effects on nitrate content in meat and vegetables where the pH and temperature are also relevant. Depending upon the heating or cooking procedure applied onto the product the nitrate content is reduced differentially as demonstrated by Prasad and Avinesh (2008); in this research, the nitrate content is highly reduced when the products are boiled in comparison with baked samples. Deep freezing has a significant effect on nitrite content but not on nitrate (Massey, 1996).

Table 1.4. Effect of storage time and pH values on residual nitrite in meat after heating

pH	Days of storage	Residual nitrite (ppm) (Ingoing = 100 ppm)	Residual nitrite (ppm) (Ingoing = 200 ppm)
5.3	0	28	70
	6	20	41
	12	5	18
5.8	0	45	120
	6	24	110
	12	13	21
6.3	0	58	135
	6	41	112
	12	31	90

Dordevic et al. (1980)

The type of meat used and the packaging technique affects residual nitrite levels. Nitrite content is highly reduced if nonfresh-frozen/thawed meat is used instead of fresh material. It seems that thawing meat allows a better penetration of the curing compounds, especially on the early stages after preparation, but the subsequent loss of nitrites during storage is lower than in fresh meat (Aksu et al., 2005). Sometimes the mechanism behind nitrite reduction is not clear. It has been seen that when different types of meat are compared, residual nitrite content is more reduced in turkey when compared with other meats like pork. This phenomenon is enhanced in the presence of high temperatures during processing (Kilic et al., 2001). Other studies concluded that nitrite reduction is increased when higher pigmentation (due to higher myoglobin content) is present, like in the case of beef and heart muscles. This increased nitrite reduction did not affect the survival of pathogens like *C. botulinum* (Tompkin et al., 1978). Also, scientific evidence correlates some modified atmosphere packaging techniques with higher nitrite decline. Reduced conditions inside meat packaging promote a higher conversion of nitrite to nitrous acid and later to nitric oxide. Vacuum packaging and the presence of 100% CO₂ has been proven to reduce residual nitrite in cured sausages (Jo et al., 2003). Oxygen and light are known factors to affect stability of cured pigments in hams and other products; oxygen at low concentrations (0.5%) and in the presence of light contributes to nitrite reduction after several days of storage (Moller et al., 2000). New approaches are currently used to reduce the content of preformed nitrosamines in some cured products. Gamma irradiation is an effective approach in reducing the content of nitrosamines and residual nitrites as well, even under aerobic packaging conditions (Ahn et al., 2002).

3.2 Monitoring of nitrite in cured products

Factors that influence nitrite chemical behavior complicate analytical quantification in cured meat products. Efforts are made in order to achieve a good correlation between residual nitrite levels, the history of the product and the implications derived from the variations. After the confirmation of the carcinogenic properties of nitrosamines and their presence in cured products in the 1960s, meat processors started changes in the curing process, including a significant reduction in the input nitrite levels and the incorporation of ascorbates (Scientific Panel on Food Additives, 2003). The final result has been a significant reduction in nitrite content in cured products.

Monitoring studies have been performed in Europe, the United States and Canada. Studies made in Germany showed that most of the tested cured products (about 50%) contained no detectable nitrite levels; in the cases where nitrite was detected the average content was around 20 mg/kg which is well below the regulatory standard of 175 mg/kg (Honikel, 2008). In Canada, from 1972 to 1996, a survey reported residual nitrite content ranging between 28 and 43.6 mg/kg in different products including bacon, ham, sausages, bologna, corned beef and others. Bologna, frankfurters and various uncooked cured products contained the highest levels of residual nitrite among the products tested (Sen and Baddoo, 1997). Low levels of residual nitrite were found in more than 70% of cured samples in Belgium in a study made from 2002 to 2003. Higher variability was found in the United Kingdom in 1997; the residual nitrite was between 0.2 and 123 mg/kg for bacon and 0.2 to 170 mg/kg for other products (Scientific panel on Food Additives, 2003). Low levels were found by Cassens in the United States (1997); most of the products tested in this study showed levels below 10 mg/kg, demonstrating the

consequences of novel curing methodologies, including lower input of sodium nitrite and the most common use of ascorbates in the last 20 years.

3.3 Technical considerations for analysis of nitrites in cured meats

The widespread occurrence of nitrites in meat, vegetables and water motivates the development of new analytical methodologies to improve technical competence during nitrite quantification. Many of these techniques are also used to determine nitrate which is present in much greater concentrations than nitrite; in contrast, the presence of nitrite is rare in most of the samples and, even in cured products, where nitrite is intentionally added, the detectable levels are very low (Massey, 1996).

Analytical problems make accurate measuring of nitrate and nitrite concentration in foods difficult. The first problem is the different ways of expressing nitrite concentration such as mg (nitrite ion)/kg, mg (nitrite as Nitrogen)/kg or mg (sodium nitrite)/kg. Another problem is the possibility of positive and negative interferences during measurement. Positive interferences in nitrite analysis are not as common as for nitrates, but they are present in many of the colorimetric assays traditionally used (AOAC, 1995). Interferences in this case include colored pigments or compounds causing turbidity inside the spectrophotometer cell. In the case of HPLC techniques, positive interferences are present when other components of the samples are eluting at the same retention time than nitrites (Massey, 1996). If nitrate is present in high quantities in the sample and the storage conditions are not adequate, nitrite is produced by bacterial reduction. Nitrite is also generated by decomposition of S-nitrosothiols during analysis (Olsman and van Leeuwen, 1977). In contrast, negative interferences are more important for nitrites than for nitrates due to the high chemical reactivity of the nitrite anion. The conjugate acid of nitrite

(nitrous acid) undergoes chemical reactions with different compounds; it has been calculated that most of the nitrite in the sample is reacting with proteins (30%), but other fractions will react with myoglobin (15%), -SH groups (15%) and lipids (15%). About 5% is present in a gaseous form and just a portion is present as residual nitrite (Honikel, 2008). Additionally, the concentration of the analyte is underestimated as a consequence of deficient extraction procedures or if physical losses (including adsorption) take place in later steps of the analytical method (Massey, 1996). Other factors are related to improper conditions during storage including low temperature (freezing conditions promote nitrite decline), bacterial contamination and mild acidic conditions (Massey, 1996). Strong alkaline conditions are recommended during storage and the sample should be extracted and analyzed as soon as possible to avoid chemical reactions of nitrite with nucleophilic species present in the sample.

In general, most of the extraction procedures take advantage of the high solubility of nitrite in water; therefore extraction in hot and cold water is used (Jimenez-Colmenero and Blazquez Solana, 2009). Other extraction protocols recommend the use of borax instead of water (British standard, 1976). The extraction procedure for nitrite analysis is not considered a main source of negative or positive interferences. The highest concern in the past during extraction is what is called the “dilution effect”. Basically, it involves the dependence of the final analyte concentration with the amount of liquid used in the extraction procedure (Usher and Telling, 1975). Most of the protocols include steps to extract the residual nitrite remaining in the sample; however, some protocols include procedures to release the fraction of nitrite bound to thiol groups in meat proteins using mercuric chloride (Olsman and van Leeuwen, 1977). Blending, filtration, centrifugation

and precipitation of proteins with Carrez reagents are typical approaches to reduce interferences. Nevertheless, Carrez reagents have been identified as a source of nitrite reduction (Rincon et al., 2003).

3.4 Analytical methods for nitrites in cured meats

New protocols for nitrite determination in meats have been developed in the last few years. Some of the methodologies are just merely variations of old techniques. The classical nitrite determination in cured meats relies in the Griess diazotization procedure where, an azo dye, is produced by coupling a diazonium salt with an aromatic amine or phenol (Zanardi et al., 2002). Most of the original colorimetric methods were based in the reaction of nitrites with sulphanilamide and N-1-naphthylethylenediamine in mildly acidic conditions, to form a red or purple dye that can be measured by a spectrophotometer (Slack, 1987) (Fig. 1.3).

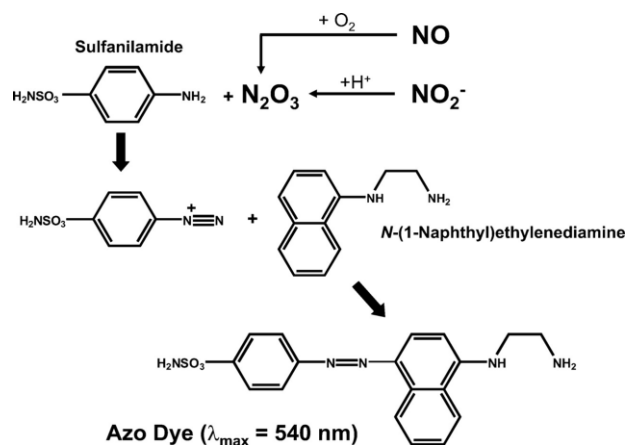


Fig. 1.3 Diazotization Coupling Reaction (Griess reaction)

High concentration of inert salts and the buffering effect of organic acids can affect the pH dependent absorbance of the azo dye (Massey, 1996). High levels of ascorbates are known to generate a negative interference by competition with the coupling reagent (Fox

et al., 1984). If the extraction was incomplete, physical contamination increases the turbidity of the sample. At the beginning, colorimetric methods were run manually and usually involved many extraction and preparation steps. Automated approaches currently available include the Technicon Autoanalyzer II[®], which allows a sequential analysis of different types of samples for nitrite content. Other laboratories work with enzymatic approaches based on the reduction of nitrates to nitrites by NADPH, in the presence of the enzyme nitrate reductase. The oxidized NADPH generates a colored compound that can be measured at 340 nm (Zanardi et al., 2002). A third colorimetric approach involves the oxidation of nitrite to nitrate with permanganate followed by acidification and treatment with *m*-xylenol. After a nitration step, nitroxylenol is removed by distillation and measured colorimetrically (AOAC, 1995). The AOAC method 973.31 specifies the protocol for extraction and measurement of nitrites in cured meats using the Griess reaction (AOAC, 1995). The extraction protocol of this method has been revised and improved recently (Mohammed et al., 2008).

Other principles for nitrite determination include chemiluminiscence, chemical titration and chromatography. Chemiluminiscence methods are characterized for having good sensitivity and high correlation with the classical colorimetric methods. In this approach nitrite is reduced by the use of ascorbic acid under acidic conditions, followed by chemiluminiscence detection of the released nitric oxide (Doerr et al., 1981). In chemical titration nitrite is reduced with Devarda's alloy under alkaline conditions to generate ammonia, which is later distilled and determined by titration (Hunt and Seymour, 1985). In the case of gas chromatography, several methods have been published since the 1970s. In chromatography, nitrites are first derivatized into a volatile compound, then extracted

into organic solvents and measured by gas chromatographer with a selective detector (Funazo et al., 1980). Recently, gas chromatography has been used to determine the levels of volatile nitrosamines in cured products (Byun et al., 2004).

Time-consuming techniques have been gradually replaced for faster methodologies including HPLC and capillary electrophoresis. Capillary electrophoresis has the advantage that can be used for simultaneous separation of anions allowing determination of nitrate and nitrite in a single step (Oztekin et al., 2002). This technique is based on the slowing down or reversal of the electroosmotic flow by buffer additives; polyethyleneimine coated capillaries can be used instead of buffers to reverse the flow (Oztekin et al., 2002). Recently, Merusi et al. (2010) published a new capillary electrophoresis technique where no buffer additives or coated capillaries are needed; basically they made the separation with buffer at very low pH values. The detection is based on UV absorbance and it has high correlation with results obtained with HPLC techniques (Jimenez-Colmenero and Blazquez-Solana, 2003).

3.5 High Performance Liquid Chromatography (HPLC) for nitrites in cured meats

HPLC is a good approach for nitrate and nitrite analysis in foods, including cured meats, vegetables, cheese, milk and water (Reece and Hird, 2000). The main advantage is the short time needed for sample preparation and analysis in comparison with the traditional methods (Zanardi et al., 2002). Particularly, ion exchange-high pressure liquid chromatography has shown applicability in the simultaneous determination of nitrate and nitrites and has no need of toxic reagents for nitrite reduction. Some of the problems associated with HPLC are complications during data interpretation, especially for interferences related with the sample's matrix and inconsistencies with the traditional

colorimetric methods (Dennis et al., 1990). Other disadvantages are that the equipment is expensive and trained analysts are required. The majority of protocols have employed UV detection methods for determine both anions but in some cases a combination of UV and visible spectrophotometry can be applied (Massey, 1996).

As nitrite's high natural chemical reactivity is a disadvantage for quantification purposes, the use of chromatography helps in the removal of contaminants and other interferences. Chromatography is just an extension of simple extraction procedures and it is based on the distribution coefficient of a molecule between two immiscible phases (Rounds and Nielsen, 2003); one of these phases is moving with respect to the other and is called the mobile phase. The static phase is called the stationary phase. Chromatographic separations were discovered in 1906 by the Russian scientist Michael Tswett (Strain and Sherma, 1967) and they have found multiple applications for chemical analysis. The basic separation principles ruling chromatography are listed in Table 5.

High Performance Liquid Chromatography (HPLC) appeared in the late 1960s as a variation and improvement of the traditional column liquid chromatography (Rounds and Gregory, 2003). HPLC improvements are related with higher resolutions and separations thanks to the application of high pressures and innovative materials inside the columns. Additionally, they can be coupled with simultaneous detection systems for quantification. Commonly, analytical HPLC columns are 10, 15 or 25 cm long with an internal diameter of 4.5 to 5 mm (Wellings, 2006). Depending on the type of separation desired and the number of theoretical plates of each column the size and length of the column may change. These columns are packed with different types of materials that serve as the support of the stationary phase in the classical liquid-liquid chromatography. For ion

exchange and affinity chromatographies the packing material may serve as both as the support and the stationary phase (Rounds and Nielsen, 2003). In the case of ion exchange techniques, the most common packing materials include macroporous functionalized organic resins that are sulfonated or aminated. Some types of columns are silica-based, where the stationary phase is bound on the surface to allow better separations (Wellings, 2006). The basic principle of ionic-exchange chromatography is a competition between the analyte and ionic molecules present in the mobile phase; by changing the ionic strength of the mobile phase the rate of how the analyte is bound or eluted can be manipulated (Larson et al., 2001). Some exceptions to this principle are found in the case of nitrate and nitrite separation methodologies in biofluids and foods. In this case, a single mobile phase is used, as the separation of the compounds is not dependent on the solution concentration (Tsikas, 2007). Differences in ionic interaction between these anions and the stationary phase are regulated by the inherent chemical structure of nitrates and nitrites' molecules, where both of them have a single electric charge of -1, but different number of sites for chemical interactions (Fig. 1.4).

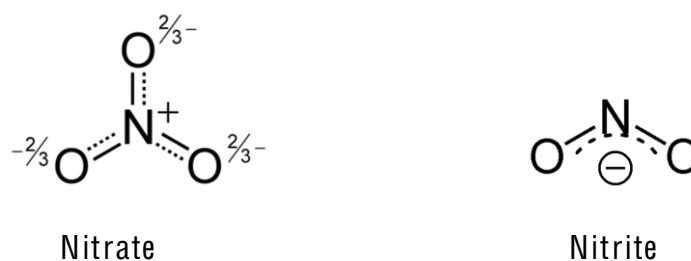


Figure 1.4. Molecular structure of nitrates and nitrites molecules

More molecular interactions are possible between nitrate and the positively charged stationary phase during HPLC analysis. Depending on the procedure, the retention time for nitrite and nitrate could be around 3 and 6 minutes respectively (Reece, 2000). A positive interference in the case of nitrite analysis is the presence of the chlorine ion because chlorine peak has a similar retention time than nitrite (Pegg, 2000). Usually, this problem is avoided by the application of appropriate extraction procedures with deionized water. The final step consists in the detection of the signal by the use of different approaches like UV/Visible spectrophotometry or refractometry (Rounds and Gregory, 2003).

Several HPLC methods have been published for the analysis of nitrates and nitrites in cured meats (Dennis et al., 1990; Wootton et al., 1985) and most of them are based on the ion exchange principle. Reverse phase HPLC has been used for some authors in cured products but the correlation between this method and ionic exchange is not considered acceptable. Jackson et al. (1984) concluded that reverse phase HPLC is not robust enough for the analysis of nitrites in cured meats when compared with ion exchange methods. The detection limits for HPLC are between 0.01 and 1.0 mg/kg in the case of some foods (Dennis et al., 1990; Eggers and Cattle, 1986). In some cases the quantification limit is as high as 2.5 mg/kg if different mobile phases or extraction procedures are used (Hsu et al., 2009). Differences in sensitivity have motivated an extensive research to compare the performance of new HPLC methods with traditional techniques. Good agreement between HPLC and colorimetric techniques for the analysis of both nitrates and nitrites in cured meats were reported by Dennis et al. (1990) and Eggers and Cattle (1986), but with the additional observation that the correlation between both methodologies is low at lower

nitrite concentrations (below 10mg/kg). Wootton et al. (1985) observed lower comparability using reverse phase HPLC. Other approach proposes the determination of nitrate and nitrite using a polymeric column. In this case, after extraction and analysis the detection limits for nitrites were between 1 and 40 mg/kg and the technique correlated well with traditional colorimetric methods (Danielson et al., 2000). Nitrites and nitrates have been determined in muscle tissue (beef, pork, horse and chicken) using ion exchange HPLC coupled with mass spectrometry detection (Jimenez-Colmenero and Blazquez-Solana, 2003).

Table 1.5. Different chromatographic methods principles

Method	Mobile/Stationary phase	Principle of Separation
Gas-liquid	Gas/liquid	Molecular size/polarity
Gas-solid	Gas/solid	Molecular size/polarity
Supercritical fluid	Supercritical fluid/solid	Molecular size/polarity
Reversed-phase	Polar liquid/non polar liquid or solid	Molecular size/polarity
Normal-phase	Less polar liquid/more polar liquid or solid	Molecular size/polarity
Ion-exchange	Polar liquid/ionic solid	Molecular charge
Size-exclusion	Liquid/solid	Molecular size
Hydrophobic-interaction	Polar liquid/non polar liquid or solid	Molecular size/polarity
Affinity	Water/binding sites	Specific structure

Adapted from Rounds and Nielsen (2003)

4. Reduction of sodium content in meat products

Sodium chloride is one of the major sources of sodium intake from diet (Desmond, 2006).

The sodium metabolism has a critical role in maintaining the metabolic homeostasis.

However, excessive sodium intake represents a serious threat to the normal metabolic activity of the organism.

4.1 General background

Sodium is a member of the alkali metals group with the symbol Na and atomic number 11. It was first isolated in 1807 by Humphrey Davy who was a British scientist and inventor, very famous for the discovery and isolation of other elements (Knight, 1992).

The accomplishment of sodium isolation is remarkable as this element does not occur naturally in nature due to its high reactivity with oxygen and water (O'Daly, 2001).

Sodium is considered an essential element and nutrient for all animal species (including humans) and for some plant organisms as well. Different sodium compounds like common salt (NaCl), soda ash (Na₂CO₃), baking soda (NaHCO₃), caustic soda (NaOH), sodium nitrate (NaNO₃), di- and tri-sodium phosphates, sodium thiosulfate (Na₂S₂O₃ · 5H₂O), and borax (Na₂B₄O₇ · 10H₂O) are important to food industry. For meat industry other products like isoascorbate or some salts of organic acids contain sodium based compounds (Desmond, 2006).

Among all the compounds containing sodium, sodium chloride has the highest importance as a source of this element in food. Salt's importance was recognized since prehistoric times and many societies through centuries disputed many wars in order to control salt production. The earliest records about salt production and war are taken from ancient Chinese literature. About 4,700 years ago Peng-Tzao-Kan-Mu published the earliest known treatise on pharmacology. A major portion of this writing was devoted to a discussion of more than 40 kinds of salt, including descriptions of two methods for extracting salt and putting it in usable form that are similar to processes used today (Salt institute). Later, western world discovered salt and the Romans were recognized as prodigious builders of saltworks. Special salt rations given to Roman soldiers were

known as "salarium argentum," the forerunner of the English word "salary" (Salt Institute, 2011). Discovery of preservative properties of salt was a crucial factor in civilization's establishment as it allowed travel over long distances. Until the 19th century salt was the most important item used for food preservation just before the refrigeration systems were invented during the First Industrial Revolution (Kurlansky, 2002). Salt consumption decreased over several decades after refrigeration development but still an important amount of salt is used for the production of modern processed foods.

There are two main sources of salt, the rock salts and seawater. Depending upon the source, the extraction procedure is different. In the case of seawater, the procedure implies a basic evaporation process using solar energy; in the cases where solar energy is not available a heating device can be used. For rock salts, mining is the best procedure and it can be physical extraction or by the use of water through a method called "solution mining" (Jacoby, 1972). Today, salt is produced all over the world but the majority of the total production is concentrated in few places. Table 6 shows a brief list of the top 5 countries in terms of salt production in the world.

Table 1.6. Countries with the highest salt production rates in the world

Country	Salt production (tonnes)	Percentage of world production (%)
World	210,000,000	100.00
United States	46,500,000	22.14
China	37,101,000	17.67
India	15,000,000	7.14
Canada	14,125,000	6.73
Australia	11,211,000	5.34

Adapted from Brown et al. (2010)

4.2 Metabolic activity of sodium

Sodium is considered an element with essential functions in the human body. This element is involved in thousands of metabolic and signaling pathways, regulating both macro and micro physiological processes (Osgood and Ocorr, 2009). Some of the most important metabolic functions attributed to sodium are listed below:

- **Fluid balance:** sodium is the main regulator of the balance of extracellular fluids by the control of crystalloid osmotic pressure in blood and lymph. Sodium concentration in blood is the first signal used by regulatory organs to modify the volume of fluids in the body.
- **Neuromuscular excitability:** like many other cations, Na^+ is involved in the transmission of nerve impulses. This is possible by the innate positive electrical charge of sodium atoms.
- **Acid-Base regulation:** sodium is interchanged by H^+ ions in the renal tubules to acidify the urine and regulate the pH of the body, especially in cases of metabolic acidosis. Together with potassium, sodium plays a role in the transportation of H^+ ions inside and outside the cells.
- **Maintenance of blood viscosity:** the interaction between sodium atoms and globulin proteins present in the blood leads to the formation of water soluble salts. Salt formation prevents these globulins from increasing blood viscosity.
- **Resting membrane potential:** Na ion concentration in extracellular fluids is far much higher than in the intracellular compartments. Plasma membranes are not permeable to Na and differences in ion concentrations generate a differential

distribution of electrical charges. The resulting membrane potential (-70 to -95 mVolts) is one of the basic factors needed for nerve impulse transmission.

To maintain these metabolic functions sodium serum concentrations should be between 135 and 145 mEq/L (Osgood and Ocorrd, 2009). Metabolic disorders are associated with alterations in sodium serum levels in the organism, including low sodium levels (hyponatremia) or high sodium levels (hypernatremia) (Rose and Post, 2001).

Hyponatremia is present when sodium serum levels are below 135mEq/L and is considered severe when the levels are lower than 125mEq/L. This metabolic disorder is usually caused by organ failure and genetic alterations, but can be initiated by the excessive consumption of diuretics as thiazides (Berl and Schrier, 2010). Common causes of hypernatremia are related with low consumption of water or high excretion of this compound, mainly by the occurrence of excessive sweating, diarrhea or genetic diseases. However, hypernatremia can be caused just by the increased consumption of sodium present in solutions of high ionic strength or diets rich in sodium content. Problems associated with high sodium levels include edema in different parts of the body, osteoporosis, renal failure, gastric cancer, asthma and obesity (He and McGregor, 2008). However, the main problem related to high sodium consumption is the elevation in blood pressure levels or hypertension.

4.3 Excessive sodium intake and high blood pressure

Fluid balance in the body is dependent on blood sodium concentration. High blood pressure is one of the consequences of excessive sodium intake from diet. Hypertension leads to cardiovascular disease (CVD) in 80% of all the cases and also is implicated in 62% of all the cases of strokes and 42% of coronary heart disease (CHD) (He and

McGregor, 2008). CVD is the first cause of death in the world and the second cause of disability, just behind the malnutrition. World Health Organization estimates that 80% of all adults are at risk of CVD from their high blood pressure and is projecting more than 26 million deaths by the year 2030 (Mackay and Mensah, 2004). The American Medical Association (AMA) estimates that CVD from high salt consumption is killing prematurely more than 150,000 persons in the United States every year. Currently, WHO is motivating a worldwide reformulation of processed and prepared foods to achieve lower levels of sodium.

A normal blood pressure is around 120/80 mm of Hg. In cases of hypertension this value could be as high as 140/90 mm of Hg or 130/80 mm of Hg in cases of persons with accompanying complications like diabetes or heart disease (Osgood and Ocorr, 2009). A value around 130/80 mm of Hg is considered as a “high normal” value for blood pressure; usually one of the first recommendations for hypertensive persons is to avoid food saturated in fat or high sodium content. The effect of sodium on the fluid balance is explained in terms of the renin-angiotensin system. In cases where blood pressure decreases, the renin-angiotensin system is activated in order to promote a higher retention of sodium in the kidneys. Most of sodium in the body is excreted at the kidney level and its retention is directly correlated with higher blood volumes, where the fluids are increased by osmotic effect. In situations where sodium levels drop (hyponatremia) the renin-angiotensin system is activated in the same way. Therefore, high sodium intake becomes an artificial way to increase sodium levels in the blood leading to increases in fluid volumes (Berl and Schrier, 2010.). If high sodium intake continues, persistent high

blood pressure leads to CVD. A more detailed explanation of the renin-angiotensin regulatory mechanism is shown in Figure 1.5.

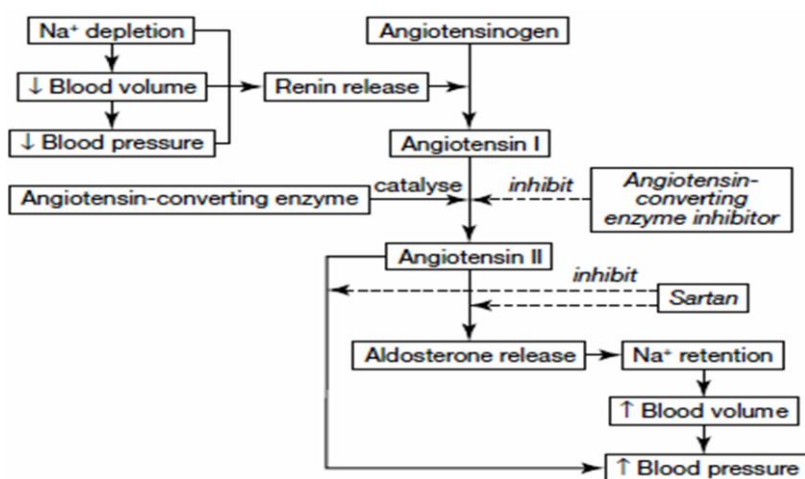


Figure 1.5. Regulation of blood pressure via sodium retention by the rennin-angiotensin system

Increased blood volumes lead to CVD by the incapacity of the cardiovascular system to support excessive pressures of fluids. Sodium has been mentioned as one important cause of hypertension worldwide and more specifically, sodium chloride is the first target to control sodium intake (Desmond, 2006). The scientific evidence establishing the relationship between hypertension and high salt intake is abundant; some of the investigations have been made in different fields like animal studies, human genetics, epidemiology, migration studies, population studies and treatment trials (He and McGregor, 2008). He and McGregor (2002) reported that all scientific studies aimed to study NaCl impact on blood pressure should be made during appropriate periods of time,

no shorter than 4 weeks; trials that fulfilled this requirement demonstrated reductions of sodium urinary excretion of 78 mmol and decreases in blood pressure of 4.96/2.73 mm Hg when NaCl consumption was controlled. In 2006, the effect of long-term reduction on normotensive individuals was analyzed; for normal persons, the reduction in blood pressure and urinary sodium excretion was similar than those observed in hypertensive individuals, supporting the evidence that salt reduction was correlated with decreases in blood pressure (He and McGregor, 2006). After 5 weeks of reduction in salt intake, decreases of 5 mm of Hg in blood pressure were reported when sodium in diet was reduced by 50 mmol (Law et al., 1999). A successful intervention study made in two villages from Portugal accomplished to determine differences in blood pressure values when salt intake levels were different between two communities. After two years of studies, the study found differences of even 13/6 mm of Hg (Forte et al., 1989). A study in Finland analyzed the link between high salt intake, blood pressure and the risk to dye of CVD. The conclusion was that an increase of 6 g/day in salt intake was related to an increase of 56% in CHD deaths and 36 % in CVD (Tuomilehto et al., 2001). Another study established a relationship between low salt intake levels and a 25 % lower incidence of any cardiovascular event after 15 years of monitoring (Cook et al., 2007). Epidemiological finding suggests that mortality for stroke is higher when urinary sodium excretion is increased (Perry and Beevers, 1992).

Other scientific papers and communications refute the link between high blood pressure and sodium intake. The Salt Institute has declared that the Center of Disease Control (CDC) is promoting a strong “anti-salt agenda” in the scientific literature aimed to reduce salt content in foods. Among the evidence presented is a paper review published in the

American Journal of Hypertension where a meta-analysis performed to evidence the relation between salt reduction and risk of CVD found no sufficient statistically power to give a conclusion; the evidence suggested that reduction of salt intake is related with increase risk of mortality for any cause (Taylor et al., 2011). Other publication made by two Harvard researchers established similar conclusions (Bernstein and Willett, 2010). The Salt Institute is intensively warning food companies not to reduce salt levels in their products before more convincing scientific evidence is presented.

One of the most recent estimates determines that in many countries the current salt intake is around 12 grams/per day/per person, with important differences depending on the region (Strazzullo et al. 2009). In the United Kingdom, the estimated daily intake of salt is 8.6 grams/person according to a recent report of the Food Standards Agency (Desmond, 2006). British government is applying strict rules in order to achieve the WHO recommendation by the year 2012. For the United States, the CDC reports establish a daily salt intake close to 9.0 grams daily, but this number could be much higher. The National Academy of Science in the USA recommends a total salt intake not higher than 6 grams per day and WHO regulations are promoting intakes even lower (around 5.5 grams per day). In Finland, where a successful program aimed to reduce salt consumption has been applied, daily sodium intake has been reduced from 14 to 8 grams per day during the last 30 years, and now the government is working to meet WHO specifications (Pietinen et al., 2008). The most important international program established to reduce salt intake is the World Action on Salt and Health (WASH), a multinational group with more than 400 members from 84 countries. This group was established in 2005 and is working to achieve salt reduction's goals at different levels including the food industry,

the governments and the consumers. Currently, thousands of products are available in the market that were formulated to be low in sodium; these products include beverages, desserts, bread, condiments, sauces, fish, fresh produce and meat products (Low salt products-Directory).

4.4 Salt, an ingredient with powerful technical functions for meat products

Sodium is part of many compounds in a typical meat product. Meat itself contains sodium in very small amounts (100 mg/100 grams of meat). Other commonly added ingredients present in formulations contain appreciable amounts of sodium like monosodium glutamate, sodium phosphates, sodium citrate, sodium lactate, sodium erythorbate and sodium nitrite (Ruusunen and Puolanne, 2005). Salt contains around 39.3% of sodium and in common meat formulations with 2% salt level, sodium from salt could be as high as 79% (Breidentsein, 1982). However, salt and sodium content can vary depending on the type of product (Table 7).

Salt control is complex as it is an ingredient with many important technical functions in food systems. For this reason, salt reduction for the meat industry is considered a challenge (Desmond, 2006) and a difficult task to achieve, but the current trend is that sodium has to be gradually reduced in most of the products. USDA has expressed the recognition of salt's importance in foods:

“Would reducing the salt content of food, even in a modest way, **impact the safety or quality** of various foods given the wide variety of technical functions for which salt is used in food? How feasible would it be to **mitigate this impact** if true? Could it be mitigated by, for example, the addition of other ingredients?”

Table 1.7. Salt and sodium content (per 100g) in typical meat products

Product	Sodium (mg)	Salt equivalent (g)
Beef patties	68	0.17
Bacon	1000-1540	2.5-3.9
Sausages	636	1.6
Frankfurters	1120	2.8
Cured ham	900-1200	2.0-3.0
Salami	1890	4.8

Food Standards Agency (2002) and USDA (2005).

Some approaches can be used in order to reduce salt content in meat products (Ruusunen and Puolanne, 2005):

1. Lowering the level of sodium chloride added, taking into account some of the issues discussed above.
2. Replacing all or some of the sodium chloride salt with other chloride salts like KCl, CaCl_2 or MgCl_2 . Potassium chloride is probably the most common salt substitute applied when formulating sodium reduced products. One of the problems when using KCl is the increase in bitterness and reduction in saltiness (Desmond, 2006). Also, the USDA Dietary Guidelines for Americans (2010) warns about the potential abuse of potassium in meat products as it can cause problems in persons with special health conditions.
3. Replacing all or some of the sodium chloride salt with non-chloride salts like phosphates or new processing techniques or modifications. At this point, salt mixtures have been probed to be effective in replacing normal salt and some of these products contain KCl, potassium lactate, calcium citrate, calcium lactate, lactose, dextrose and ascorbic acid (Riera et al., 1996). Phosphates are also a good option to replace water-holding capacity of salt.

4. Combination of any of the other approaches. New techniques are applied in meat products that can support the function of other salt replacers. One approach is the development of new physical forms for salt crystals; it helps to increase the sensation of saltiness and lower amounts of salt are needed (Angus et al., 2005). Some authors suggest that research should also be directed towards the meat itself, applying methods that will increase functionality of the products; two examples of this are the use of pre-rigor meat and high-pressure technology during processing (Claus and Sorheim, 2006).

The search for a complete substitute of salt is still active. In modern meat industry, salt is used as a flavoring enhancer, modifier of meat texture, it activates proteins to increase hydration and water-bonding capacity, increases viscosity of meat butters and is a bacteriostatic agent (Terrell, 1983). Flavor properties of salt are the first property to be analyzed when product formulations are modified. Perceived saltiness is a phenomenon produced by the chemical interaction of Na^+ ion with sensory receptors in the tongue; the overall sensation is modified by the presence of Cl^- (Ruusunen and Puolanne, 2005).

Saltiness sensation depends on the type of product tasted. Evidence has been found that increase in saltiness is more noticeable when the meat product has a high content of fat (Ruusunen et al., 2001). The effect of meat on saltiness is intensive, and higher amounts of salt are needed to increase saltiness of products with high meat content. These aspects must be taken into account when evaluating sensory properties of new developed products. For example, in the specific case of hams, some reports say that reduction of salt can lead to higher losses of saltiness because the fat content in hams is not as high as other products (like sausages) (Frye et al., 1986; Lin et al., 1991). Saltiness is very

important for consumers and salt reduction should be accomplished gradually in order to not negatively affect consumer's acceptance of new meat products (Bertino et al., 1982). The advantage is that consumer's health is not compromised and saltiness threshold levels are decreased, as the sensory receptors in the tongue get used to the new salt levels in diet.

By increasing water-holding capacity, meat products retain tenderness and juiciness and the cooking process does not affect the quality (Desmond, 2006). Hamm (1986) explained the mechanism of interaction between NaCl and myofibrillar proteins in meat; it is understood that chloride ion is more strongly bound to proteins than Na and it causes protein molecules to get a negative charge. Repulsion between molecules results in swelling of myofibrils and loosening of general matrix. Salt presence shifts isoelectric point to lower pH values, making interaction between molecules with opposite charges weaker at higher pH. The overall effect is that proteins get solubilized forming a sticky exudate on the surface of meat pieces, helping to maintain the product together. Other additional benefit is the attachment of fat inside the protein film formed after solubilization (Monahan and Troy, 1997).

4.5 Antimicrobial properties of salt

Antimicrobial properties of salt were the oldest feature manipulated by prehistoric civilizations in order to preserve foods during a time where refrigeration was not available (Kurlansky, 2002). The early application of salt for food preservation allowed the discovery of the curing process that is still used today. However, antimicrobial capacity of salt is not the first aspect noticed by consumers' and general public; other features, like taste, are considered of higher impact. But, for meat processors, new

formulations aimed to reduce salt content are extensively tested in terms of microbial safety, as NaCl helps to control the occurrence of pathogenic and spoilage microorganisms. Salt is considered the most effective and versatile antimicrobial applied in foods as it has the capacity to affect any microorganism present in food (Baros J. A., 2001). Salt is more effective than refrigeration to control microbial growth as some species like *Listeria monocytogenes*, *Yersinia enterocolitica* and *Pseudomonas* spp. are capable to grow at refrigeration temperatures (5°C) (Montville and Mathews, 2008). Antimicrobial capacity of salt mostly relies in the reduction of the water activity in the medium (A_w). Water activity is defined as the vapor pressure of a liquid divided by that of pure water at the same temperature; basically it refers to the movement of water particles and how this is affected by the presence of some solutes (Fenemma et al., 2008). The higher the concentration of solute, the lower the movement of water particles. In the presence of highly concentrated solutions a mechanism of chemical compensation promotes the efflux of water from inside the cell to the outside in a process facilitated by the presence of a semi-permeable membrane (Sherwood, 2010). Bacterial metabolic pathways start an increased activity to synthesize macromolecules for equilibrating solute concentrations at both sides of the membrane. This process requires the application of high amounts of energy and the cell growth is inhibited (Montville and Mathews, 2008). If water flow is high, the shrinkage of the cell promotes cell death by plasmolysis (Montville and Mathews, 2008). Usually, a water activity value of 0.920 is inhibitory for all pathogenic species in foods (Tapia et al., 2007). Other compounds present in the media can reduce water activity. The chemical nature of the compound accounts for an important efficacy of the inhibitory power, even if A_w values are the same.

Antimicrobial properties of NaCl go beyond a dehydration process. It seems that microbial inhibition of NaCl is a direct effect of Cl^- toxicity, removal of oxygen, increased sensitization to CO_2 and reduced activity of proteolytic enzymes (Taormina, 2010). NaCl is more inhibitory than other solutes at the same A_w value (Table 8).

Some bacterial species have developed special strategies to tolerate low water activity values including some foodborne pathogens like *Listeria monocytogenes*, *Staphylococcus aureus* and *Vibrio parahaemolyticus* (Taormina, 2010). For *L. monocytogenes* the minimal A_w for growth is 0.930 and in the case of *S. aureus* is 0.860 (Montville, 2008). Other resistance forms like spores can resist low water activity conditions (Staack et al., 2008). Microorganisms that are dependent of high NaCl concentration or other ions to survive are halophiles, and if they can grow at high concentration of organic solutes they are osmophiles. Extreme halophiles can grow in salt concentrations as high as 30% (Baros, 2001). Non-osmophile species can develop higher resistance to saturated salt solutions by the activation of general stress responses, regulated by specific genes when the cell is exposed to mild inhibitory conditions (Tapia et al., 2007). In the case of spoilage microorganisms, yeast and molds are the most relevant group, especially for foods with elevated sugar concentrations (Baros, 2001). In general, specific pathogens and spoilage microorganisms are related with different foods depending on the salt concentration they can tolerate (Table 9).

The capacity of any microorganism to survive under specific A_w conditions is also influenced by intrinsic factors in food like pH, redox potential, presence of other antimicrobial compounds, storage temperature and heat treatment (Montville and Mathews, 2008). In the case of reduced salt products, food safety is not compromised if

other factors in the food matrix are controlled. However, any change made in salt levels should be coupled with the experimental confirmation that the safety of the product is not reduced (Stringer and Pin, 2005).

Table 1.8. Influence of solute on minimal inhibitory A_w values

Species	NaCl	Glucose	Glycerol
<i>Clostridium perfringens</i>	0.970	0.960	0.950
<i>Clostridium botulinum</i>	0.970	-----	0.940
<i>Lactobacillus helveticus</i>	0.963	0.966	0.928
<i>Streptococcus lactis</i>	0.965	0.949	0.924
<i>Pseudomonas fluorescens</i>	0.957	-----	0.940
<i>Vibrio parahaemolyticus</i>	0.948	0.984	0.937

Sperber (1983).

Table 1.9. Typical bacterial species associated with foods containing different salt levels.

Food type	Salt level (%)	Spoilage bacterium	Pathogens
Marine fish	1-4	<i>Pseudomonas</i> spp., <i>Shewanella putrefaciens</i> , <i>Moraxella</i> spp.	<i>V. cholerae</i> , <i>C. perfringens</i> , <i>Salmonella</i> spp.
Brined meats	1-7	Predominantly gram positive bacteria in vacuum packages	<i>C. perfringens</i> , <i>C. botulinum</i> , <i>S. aureus</i>
Salted vegetables	1-15	Moderate halotolerant molds and gram positive bacteria	Usually <i>S. aureus</i> in high salted vegetables
Salted fish	20-25	<i>Halobacterium</i> spp., <i>Halococcus</i> spp.	<i>S. aureus</i>

Adapted from Baros (2001).

4.6 Microbiological studies in reduced salt products

Several studies have been made to demonstrate the effect of salt reduction in the microbiological stability of meat products. Devlieghere et al. (2009) studied the effect of a combination of lactate and diacetate salts on shelf life of canned ham when salt was

reduced. They reported that salt can be reduced even a 40% in the presence of the antimicrobial mixture without compromising the typical shelf life of the product. Bozianis et al. (2007) concluded that equimolar concentrations of NaCl and KCl have the same effect on *L. monocytogenes* growth in a broth system, using different pH values and nisin concentrations. In other study, a combination of 1% NaCl, 0.55% KCl and 0.74 % CaCl₂ did not affect the microbial stability or safety of dry fermented sausages in comparison with a control sample with 2.6% NaCl. However, a 50% substitution of NaCl with KCl and MgCl₂ reduced the time for toxin production of *C. botulinum* in cured turkey frankfurters held at 27°C (Barbut et al., 1986). In turkey frankfurters formulated with less than 2.5% of NaCl different doses of gamma radiation were not sufficient to reduce *C. botulinum* toxin production (Barbut et al., 1987).

Zaika (2003) assessed the effect of sodium chloride on *C. perfringens*. This author concluded that sodium chloride levels below 2% and, in the absence of other antimicrobial approaches, would promote a high outgrowth of *C. perfringens* in both cooked boneless ham and roast beef during abusive cooling conditions. In vacuum packed turkey products the D-values determined at 15°C for *C. perfringens* were 27.2 min in products with 0% of salt and 17.7 min in products with 3% of NaCl (Juneja and Marmer, 1996).

5 Use of organic acids for food preservation

5.1 General information

Organic acids are organic compounds with acidic properties that are naturally present as constituents of foods (Gomis, 1992). They are weak acids and their acidity is conferred by

the presence of the –OH, -SH, enol group and phenol group (Theron and Rykers, 2011). As other organic compounds they have the common characteristic of having carbon in their molecules. Organic acids can be present in two forms: pure acids or buffered acids (Theron and Rykers, 2011). Pure acid is the typical molecular form of lactic, acetic, benzoic or citric acids. The buffered acids are the calcium or sodium salts of these compounds which are commonly used in food processing because they are safer to use, less caustic to machinery, and more soluble in water solutions (Hoffman and Possin, 2000). The typical molecular structure of organic acids includes the saturated straight-chain monocarboxylic acids structure or its derivatives. Typically, these compounds are referred to as fatty acids, volatile fatty acids, weak acids or carboxylic acids (Ricke, 2003). Members of the organic acid group present differences in molecular structure including number of carbon atoms, level of saturation, number of hydroxy groups and presence of specific functional groups (Permprasert and Devahastin, 2005). Molecular differences account for different functions and properties in food systems.

Among the most relevant chemical properties of organic acids are the pKa value, the partition coefficient and the solubility. The pKa value is defined as the logarithmic expression of the dissociation equilibrium of an acidic molecule in a water solution (Fennema, 2008). Organic acids do not fully dissociate into ions and establish equilibria between the dissociated and undissociated forms of the molecule (Stratford and Eklund, 2003). The pKa is also defined as the pH value where the concentrations of both the dissociated and undissociated form of the acid are at the same concentration (Stratford and Eklund, 2003). This value is characteristic of every organic acid (Table 10). The lower the pH of the solution the higher the concentration of the undissociated form of the

molecule. Solubility also varies depending on the identity of the organic acid. Many organic acids are characterized for being large hydrophobic molecules with positive partition coefficients and poor solubility in water solutions (Stratford and Eklund, 2003). Chemical properties of organic acids are highly dependent on the molecular structure.

Table 1.10. General characteristics of some typical organic acids in foods.

Organic acid	Formula	pKa	Foods where this acid is present
Acetic acid	CH ₃ COOH	4.76	Vinegars, pickles, salad dressings
Ascorbic acid	C ₆ H ₈ O ₆	4.10	Jams and jellies, drinks, vegetables
Benzoic acid	C ₇ H ₆ O ₂	4.20	Mayonnaise, fruits, alcoholic drinks
Butyric acid	C ₄ H ₈ O ₂	4.83	Butter, whipping cream, cheese
Formic acid	CH ₂ O ₂	3.75	Mustard, pickles, fish products
Lactic acid	C ₃ H ₆ O ₃	3.83	Dairy products, salad dressings
Propionic acid	C ₃ H ₆ O ₂	4.87	Bread, flour, tomato puree
Pyruvic acid	C ₃ H ₄ O ₂	2.39	Red apples, onions, red wine
Sorbic acid	C ₆ H ₈ O ₂	4.76	Drinks, bakery products, fish

Adapted from Stratford and Eklund (2003) and Theron and Rykers (2010)

5.2 Regulation and health concerns

Few health problems in humans related to organic acids have been reported. Sodium benzoate is known to produce hyperactivity of tight chests in individuals suffering with asthma (Motala and Steinman, 2008). Sodium benzoate in combination with sodium sorbate can generate pro-oxidant effects in humans (Theron and Rykers, 2011). However, the pro-oxidant mechanism of some organic acids remains still unclear and should be further investigated (Piper, 1999). With some exceptions, there are no limits for acceptable daily intake of organic acids for humans (Doores, 2005). Sodium diacetate recommended daily intake is between 0 and 15 mg/kg of bodyweight.

Most of the limitations in terms of organic acids application are related to loss of quality and lower consumer's acceptance of the products. In the specific case of the meat industry, the need for further antimicrobial treatments to reduce the contamination of

carcasses is motivating regulatory agencies in Europe to include organic acids as part of the regulation for slaughterhouses (Smulders and Greer 1998). In the United States the FDA allows the use of preservatives such as organic acids only as an additional antimicrobial approach in acidic foods. The FDA includes different types of organic acids in the list of GRAS substances (FDA, 2011), like acetic and lactic acids, and some related compounds like calcium lactate. There are no specific regulations other than those established by good manufacturing practices regarding the maximum limit that can be used. The FSIS establishes regulations in terms of permissible levels of organic acids and their salts in meat and poultry products. In the case of potassium and sodium salts of lactic acid, the recommendation is a maximum level of 4.8 % of weight of the total formulation; for sodium diacetate, the regulation establishes a limit of 0.25 % of the total weight formulation (USDA-FSIS, 2000).

5.3 Applications of organic acids in foods

Many organic acids are approved as food additives by USDA legislation and they are added to foods for different purposes (Stratford and Eklund, 2003). Organic acids are commonly used as acidulants, preservatives, antioxidants, flavors or emulsifiers. Some compounds can present some of these attributes simultaneously. For example, acetic acid has GRAS status and is used worldwide as a food additive and preservative (Smulders and Greer, 1998). It improves the flavor of different products like salad dressings and meats and also enhances the flavor impaired by other additives. The salts of acetic acid (sodium acetate and sodium diacetate) are also used to improve flavor and preserve foods (Jensen et al., 2003). Citric acid is also commonly used as a preservative and acidulant (Marz, 2002). The fresh acidic flavor of citric acid makes it appropriate for improving the

taste of different products. One of the advantages of citric acid is its high solubility in water solutions, which makes possible its application in both food and pharmaceutical industries (Theron and Rykers, 2011). Lactic acid is considered a GRAS substance as well with multiple applications; it is used as an acidulant, flavoring agent, pH buffering agent and preservative (Valli et al., 2006). Lactic acid increases flavor and aroma and is a strong microbial inhibitor. In fact, around 85 % of all lactic acid produced in the United States is used in some type of food-related application (Zhang et al., 2007). The sodium salt of lactic acid (sodium lactate) has proven to be effective as an oxidant and color stabilizer of cooked beef (Choi and Chin, 2003); lactates are also used as humectants because their hygroscopic nature is used to bind water in the food matrix to prevent product desiccation (Shelef, 1994). Other compounds like malic acid are mostly used as preservatives; however, due to its smooth lingering taste it is also applied to mask the bitter aftertaste of synthetic sweeteners (Theron and Rykers, 2011). Organic acids are also beneficial for perfume industry, pharmaceuticals, medicines and the production of other chemicals (Theron and Rykers, 2011).

5.4 Antimicrobial activity of organic acids

Organic acids inhibit microbial growth and are considered bacteriostatic compounds (Doores, 2005). They are the most extensively used preservatives in the food industry (Plumridge et al., 2004). They have shown antimicrobial activity to control the presence of pathogenic and spoilage microorganisms, especially bacteria, yeasts and molds (Stratford and Eklund, 2003). Organic acids have been extensively used as preservatives since ancient times as they are common constituents of foods and a normal product of

microbial metabolism (Theron and Rykers, 2011). These compounds have shown to be effective under a wide variety of processing conditions.

One of the main advantages of organic acids is their small molecular size and simple molecular structure. It enables them to move freely throughout the microbial membranes to release their antimicrobial machinery in the cytoplasm (Theron and Rykers, 2011). Once inside the cell, organic acids have the capacity to alter internal cytoplasmic pH, cell wall stability, membrane constitution and general metabolism (Stratford and Eklund, 2003). However, the most obvious antimicrobial effect from organic acids is the acidification of the external medium. Lowering the pH prevents germination of spores and growth of vegetative cells of most microbial species (Smelt et al., 1982). Pathogenic bacteria are more affected by lower pH values than spoilage microorganisms. Usually, yeast and molds are able to survive under high acidity conditions. Weak organic acids show higher inhibitory power than strong acids at the same pH levels because other antimicrobial mechanisms are functioning together.

Organic acids establish pH-dependent equilibria between the anions and the undissociated form of the molecules. The undissociated form of the molecule can easily pass through cell membranes due to the absence of electrical charges. At the higher pH of the cytoplasm (usually around 6.5) the molecule releases the proton molecule (H^+) acidifying the internal environment of the microorganisms (Krebs et al., 1983). The final result is a complete disturbance of bacterial metabolism by the acidic conditions (Stratford and Eklund, 2003). This phenomenon has been identified as the “weak acid theory” and is specific for organic acids (Paulsen and Smoulders, 2003). Passing through microbial membranes is particularly relevant for small molecules like formic, acetic, propionic,

butyric and benzoic acids (Lohmeier-Vogel et al., 1998). The rate on how the concentration of the undissociated form of the organic acid increases depends on the media pH and the respective pKa. The accumulation of the anionic form of the organic acids can also have a toxic effect on internal metabolism by an increase in the cytoplasmic osmotic pressure (Breidt et al., 2004). A general disturbance of microbial metabolism affects other stress responses like heat-shock or oxidative responses (Piper et al., 2001). For example, the presence of acetic acid significantly increases the sensitivity to heat in bacteria exposed to this compound (Lueck, 1980). Other organic acids act at the membrane level. That is the case of saturated, lipophilic molecules that can chemically interact and be accumulated in the cell membrane (Gershon and Shanks, 1979). Compounds present in the cell membrane alter membrane fluidity and permeability and some of the transportation mechanisms established at this level (Stratford and Eklund, 2003). Additional mechanisms outside the cell are related to the anionic nature of most of the organic acids. Metal chelation is a relevant inhibitory mechanism where organic acids interact with cations such as Ca^+ and Fe^+ , not making them available for microbial nutrition (Stratford and Eklund, 2003). Metal chelation properties of some organic acids like citric, succinic and lactic acid have been established (McColl and Pohlman, 1986). This mechanism is especially relevant for di- and tricarboxylic acids (Paulsen and Smoulders, 2003). However, precipitation of metallic complexes due to the presence of organic acids is a disadvantage in terms of food quality.

Small concentrations of diluted organic acids are usually needed for preservation without affecting quality attributes of the food. The combination of organic acids allows for reduction of the added concentrations without compromising safety and stability. Organic

acids are more effective when used in combination; however, limited information is available regarding the antimicrobial effect of these mixtures (Theron and Rykers, 2011). Lactic acid is a particular case because its antimicrobial capacity is considered lower than other antimicrobial acids, but has the advantage that it is active even at neutral pH levels (Houtsma et al., 1993). The antimicrobial mechanism of lactic acid and lactates is not clear. The hydrophilicity of the lactate molecule makes it difficult to move through the plasma membrane and to acidify the cytoplasmic pH (Suomalainen and Oura, 1955). The principal antimicrobial mechanism of lactic acid seems to be the acidification of the external pH. However, other mechanisms such as metal chelation (particularly Fe^{3+}) and reduction of water activity can contribute to the overall inhibitory effect (Stratford and Eklund, 2003). The capacity of lactic acid to reduce water activity has been confirmed (Abou-Zeid et al. 2007). This compound can bind water molecules with great affinity making them less available for microbial growth (Paulsen and Smoulders, 2003).

5.5 Scientific studies involving organic acids

A variety of organic acid combinations including acetic acid or its derivatives have been used to decontaminate meat and poultry products. A concentration of 2 % of acetic acid reduced the incidence of *Salmonella* spp. on pork cheek meat and significantly improved the general microbial quality of the product (Frederick et al., 1994). Acetic acid at 1.5% or 3% produced minimal reductions in populations of *E. coli* O157:H7, *Listeria innocua* and *Clostridium sporogenes* on beef carcasses stored at 5°C during 21 days (Dorsa et al., 1997). Also, in beef carcasses it has been demonstrated that acid-adapted *E. coli* O157:H7 strains can survive spray treatments with a 2% acetic acid solution (Berry and Cutter, 2000). Acetic acid present in the composition of buffered vinegar (2.5 %) can control the

germination and outgrowth of *C. perfringens* in ground turkey meat after 21 h of abusive cooling (Valenzuela-Martinez et al., 2010). As acetic acid is more inhibitory than other organic acids (Vasseur et al., 1999), the same study demonstrated that higher concentrations of the ingredient are needed (3.5 %) if acetic acid is combined with citric acid (buffered vinegar plus lemon juice).

The antimicrobial efficacy of lactic acid on beef carcasses has been studied. Kang et al. (2001) observed that a 2% lactic acid solution applied onto beef carcasses promoted significant reductions in microbial counts of aerobic, psychrotrophic and lactic acid bacteria. Similarly, a 2% solution of lactic acid applied with hot water has shown to be effective in reducing *E. coli* O157:H7 and *S. Typhimurium* populations in beef trimmings (Ellebracht et al., 1999). In pork carcasses, hot solutions of lactic acid at 2% or 5 % are effective in decontaminating pork carcasses inoculated with *S. Typhimurium* (van Netten et al., 1995). Cold tolerant pathogens like *Listeria monocytogenes*, *Yersinia enterocolitica* and *Aeromonas hydrophila* were reduced with a 3% lactic acid solution applied on pork fat and lean tissue (Greer and Dilts, 1995). Lactic and acetic acids from microbial metabolism of lactic acid bacteria (LAB) can inhibit the growth of *C. perfringens*, *E. coli* and *S. Typhimurium* by reduction of the pH in chicken feed media (Murry et al., 2004).

Despite the antimicrobial role of lactic and acetic acids in meat products, their application can lead to negative effects on quality and sensory attributes. Discoloration and development of off-flavors are associated with concentration of organic acids that are needed to achieve microbial inhibition (Smulders et al., 1986). Lower impact on quality is associated with salts of organic acids. Jensen et al. (2003) found that combinations of lactate and diacetate salts added on pork chops decreased the rate of discoloration,

increased tenderness and enhanced juiciness. Additionally, the product had lower microbial counts. As similar antimicrobial activity is expected from salts of organic acids (Theron and Rykers, 2011) some processors prefer the addition of these compounds instead of the pure form of the molecule. Little is known about the antimicrobial role of organic acids salts, but scientific evidence confirms that antimicrobial efficacy is enhanced when they are applied in combination. Combinations of sodium lactate and sodium diacetate are considered to be the most important strategies to control *Listeria monocytogenes* in ready-to-eat meat products (Theron and Rykers, 2011). Lactates are applied in a variety of meat products but their antimicrobial activity is higher in cured and cooked products (Smulders and Greer, 1998). Sodium lactate at a 3-4% level could effectively control *L. monocytogenes*, *E. coli* O157:H7 and *S. Typhimurium* in cooked beef (Miller and Acuff, 1994). In some circumstances, sodium lactate can increase the heat resistance of *L. monocytogenes* and the addition of sodium diacetate has shown to be important to increase antimicrobial efficacy (Juneja, 2003). Also, toxin formation by *C. botulinum* is retarded by different concentrations of sodium lactate in comminuted raw turkey (Maas et al., 1989). When applied in cooked ground beef, sodium lactate (2 %) alone or in combination with some spices was not effective in controlling *C. perfringens* growth after 21 h of abusive chilling (Sabah et al., 2004). In beef goulash stored under abusive temperature conditions, a solution of calcium lactate (1.5 %) had higher inhibitory activity than sodium lactate in controlling *C. perfringens* and *B. cereus* outgrowth (Aran, 2001). Velugotti et al. (2007) made similar conclusions after analyzing the effect of different lactate salts on *C. perfringens*.

In the case of sodium diacetate, it was shown that a 0.2 % concentration is enough to inhibit *L. monocytogenes* in cooked ham stored at 4°C for 40 days (Stekelenburg and Kant-Muermans, 2001). Mbandi and Shelef (2001) found that similar sodium diacetate concentrations have little effect on pH and sensory characteristics of meat. Sodium diacetate at a concentration of 0.25 % can inhibit *C. perfringens* germination and outgrowth in roast beef subjected to abusive cooling conditions (Sabah et al., 2003). However, in some circumstances sodium diacetate can decrease the inhibitory effect of some ingredients; it was demonstrated by Thippareddi et al. (2003) that sodium diacetate in combination with buffered sodium citrate is less effective than buffered sodium citrate alone to control *C. perfringens* outgrowth under abusive chilling conditions. Sodium lactate or sodium acetate alone was more effective to control *C. perfringens* outgrowth in turkey breast than buffered sodium citrate (Juneja and Thippareddi, 2004b). A combination of sodium lactate and sodium diacetate (2%) greatly enhanced the microbial stability of roast beef inoculated with *C. perfringens* even after 21 h of abusive chilling (Juneja and Thippareddi, 2004a; Sanchez-Plata et al., 2005). Similarly, a 3% combination of potassium lactate and sodium diacetate increased the microbial stability of cured cooked ham by reducing the growth of indigenous lactic acid bacteria (Devlieghere et al., 2009).

6. References

- Abou-Zeid K. A., Yoon K. S., Oscar T. P., Schwarz J. G., Hashem F. M. and Whiting R. C. 2007. Survival and growth of *Listeria monocytogenes* in broth as a function of temperature, pH and potassium lactate and sodium diacetate concentrations. J. Food Prot. 70: 2620-2625.
- Ahn H. J., Kim J. H., Jo C., Lee C. H. and Byun M. W. 2002. Reduction of carcinogenic N-nitrosamines and residual nitrite in model system sausage by irradiation. Food Chem. And Toxic. 67: 1370-1373.

- Aksu M. I., Kaya M. and Wood H. 2005. Effect of modified packaging, storage period and storage temperature on the residual nitrate of sliced-pastirma, dry meat product, produced from fresh meat and frozen/thawed meat. *Food Chem.* 93: 237-242.
- Alouf J. and Popoff M. 2006. *Bacterial Protein Toxins*. California, Elsevier. Page 773.
- Amezquita A., Weller C. L., Wang L, Thippareddi H. and Burson D. 2005. Development of an integrated model for heat transfer and dynamic growth of *Clostridium perfringens* during the cooling of cooked boneless ham. *Int. J. Food Microb.* 101: 123-144.
- Andersson A., Ronner U. and Granum P. 1995. What problems does the industry have with the spore-forming pathogens *Bacillus cereus* and *Clostridium perfringens*? *Int. J. Food Microb.* 28: 145-155.
- Ando X., Tsuzuki T., Sunagawa H. and Oka S. 1985. Heat resistance, spore germination and enterotoxigenicity of *Clostridium perfringens*. *Microb. Immun.* 29: 317-326.
- Angus F., Phelps T., Clegg S., Narain C. den Ridder C. and Kilcast D. 2005. Salt in processed foods: Collaborative Research Project. Leatherhead Food International.
- Aran N. 2001. The effect of calcium and sodium lactates on growth from spores of *Bacillus cereus* and *Clostridium perfringens* in a 'sous-vide' beef goulash under temperature abuse. *Int. J. Food Microb.* 63: 117-123.
- Association of Official Analytical Chemists Official Method. 1995. Nitrites in cured meat. 973.31. 18th edn, AOAC, Arlington.
- Barbut S., Maurer A. J. and Thayer D. W. 1987. Gamma-irradiation of *Clostridium botulinum* inoculated turkey frankfurters formulated with different chloride salts and polyphosphates. *J. Food Sci.* 52: 1137-1139.
- Barbut S., Tanaka N. and Maurer A. J. 1986. Effects of varying levels of chloride salts on *Clostridium botulinum* toxin production in turkey frankfurters. *J. Food Sci.* 51: 1129-1131.
- Baros J. A. 2001. Halophilic and Osmophilic Microorganisms. *Compendium for methods for the microbiological examination of foods* (4th ed.). Pouch F. Washington D.C., American Public Health Association. Pages 187-190.
- Benjamin N. and Collins J. 2003. *Food Preservatives* (2 ed). Russell N. and Gould G (Eds.). New York, Kluwer Academic. Page 102.
- Bergey D. and Holt H. 1994. Endosporeforming Gram positive rods. *Bergey's Manual of Determinative Bacteriology*. Philadelphia, Lippincott Williams and Wilkins. Page 561.
- Berl T. and Schrier R. W. 2010. Renal and electrolyte disorders. Philadelphia, Lippincott Williams and Wilkins. Page 20.
- Bernstein A. M. and Willett W. C. 2010. Trends in 24-h urinary sodium excretion in the United States, 1957-2003. A systematic review. *Amer. J. Clin. Nutr.* 92: 1172-1180.

- Berry E. D. and Cutter C. N. 2000. Effect of acid adaptation of *Escherichia coli* O157:H7 on efficacy of acetic acid spray washes to decontaminate beef carcass tissue. Appl. Env. Microb. 66: 1493-1498.
- Bertino M., Beauchamp G.K. and Engelman K. 1982. Long-term reduction in dietary sodium alters the taste of salt. Am. J. Clin. Nutr. 36: 1134-1144.
- Bozariis I. S., Skandamis P. N., Anastasiadi M. and Nychas G. J. 2007. Effect of NaCl and KCl on fate and growth/no growth interfaces of *Listeria monocytogenes* Scott A at different pH and nisin concentrations. J. Appl. Microb. 102: 796-805.
- Bozkurt H. and Erkmen O. 2004. Effect of nitrate/nitrite on the quality of sausage (sucuk) during ripening and storage. J. Sci. Food Agric. 84: 279-286.
- Breidenstein B. C. 1982. Understanding and calculating the sodium content of your products. Meat Processing. 21: 62.
- Breidt F., Hayes J. S. and McFeeters R. F. 2004. Independent effects of acetic acid and pH on survival of *Escherichia coli* in simulated acidified pickle products. J. Food Prot. 67: 12-18.
- British Standard. 1976. Meat and meat products. Part 7, determination of nitrate content. Part 8, determination of nitrite content. BS 4401. The British Standards Institute, London.
- Brown T. J., Bide T., Hannis S. D., Idoine N. E., Hetherington L. E., Shaw R. A. et al. 2010. World Mineral Production 2004-2008. United Kingdom, NERC. Page 86 and 87.
- Buchanan R. L. and Solberg M. 1972. Interaction of sodium nitrite, oxygen and pH on growth of *Staphylococcus aureus*. J. Food Sci. 37: 81-85.
- Byun M. W., Ahn H. J., Kim J. H., Lee J. W., Yook H. S. and Han S. B. 2004. Determination of volatile N-nitrosamines in irradiated fermented sausages by gas chromatography coupled to thermal energy analyzer. J. Chrom. A. 1054: 403-407.
- Cambridge University Press. 2011. Cambridge Dictionary Online. Available at http://dictionary.cambridge.org/dictionary/british/cure_2. Accessed on July 2, 2011.
- Carman R., Sayeed S., Li J., Genheimer C., Hiltonsmith M., Wilkins T. and McClane B. 2008. *Clostridium perfringens* toxin genotypes in the feces of healthy North Americans. Anaerobe. 14: 102-108.
- Carpenter C. E., Reddy D. and Cornforth D. P. 1987. Inactivation of clostridial ferredoxin and pyruvate-ferredoxin oxidoreductase by sodium nitrite. Appl. Env. Microb. 53: 549-552.
- Cassens R. G. 1997. Residual nitrite in cured meats. Food Technol. 51: 53-55.
- Cassens R. G., Izumi K., Lee M., Greaser M. L. and Lozano J. 1981. N-Nitroso compounds. Scanlan R. and Tannenbaum S (Eds.). Washington D. C., American Chemical Society. Pages 289.

- Choi S. H. and Chin K. B. 2003. Evaluation of sodium lactate as a replacement for conventional chemical preservatives in comminuted sausages inoculated with *Listeria monocytogenes*. Meat Sci. 65: 531-537.
- Chow C. K. 2008. Consumption of cured meats and risk of obstructive pulmonary disease. Am. J. Clin. Nutr. 88: 1703-1707.
- Christiansen L. N., Johnston R. W., Kautter D. A., Howard J. W. and Aunan W. J. 1973. Effect of nitrite and nitrate on toxin production by *Clostridium botulinum* and on nitrosamine formation in perishable canned comminuted cured meat. Appl. Microb. 25: 357-362.
- Christiansen L. N., Tompkin R. B. and Shaparis A. B. 1978. Fate of *Clostridium botulinum* in perishable canned cured meat at abuse temperature. J. Food Prot. 41: 354-355.
- Claus J. R. and Sorheim O. 2006. Preserving pre-rigor meat functionality for beef patty production. Meat Sci. 73: 287-294.
- Code of Federal Regulations. 2005. Title 21, Volume 3, Food and Drugs. Part 170, Food Additives, Sections 170.60, 172.170, 172.175.
- Cook N. R., Cutler J.A., Obarzanek E., Buring J. E., Rexrode K. M., Kumanyika S. K. et al. 2007. Long term effects of dietary sodium reduction on cardiovascular disease outcomes: observational follow-ups of the trials of hypertension prevention. BMJ. 334: 885.
- Crouch E. and Golden N. 2005. A risk assessment for *Clostridium perfringens* in ready-to-eat and partially cooked meat and poultry products. USDA.
- Danielson N. D., Sherman J. H. and Schomaker D. D. 2000. Food Analysis by HPLC (2nd ed.). Nollet L. M (Ed.). New York, Marcel Decker Inc. Page 996.
- de Man J. M. 1999. Principles of Food Chemistry. Maryland, Aspen Publishers. Page 386.
- de Oliveira T. L., de Araujo S. R., Ramos E. M., das Gracas C. M., Alves E. and Piccoli R. H. 2011. Antimicrobial activity of *Satureja montana* L. essential oil on *Clostridium perfringens* type A inoculated in mortadela-type sausages formulated with different levels of sodium nitrite. Int. J. Food Microb. 144: 546-555.
- Dennis M. J., Key P. E., Papworth T., Pointer M. and Massey R. C. 1990. The determination of nitrate and nitrite in cured meat by HPLC/UV. Food Addit. Contam. 7: 455-461.
- Desmond E. 2006. Reducing salt: A challenge for the meat industry. Meat Science. 74: 188-196.
- Devlieghere F., Vermeiren L., Bontenbal E. Lamers P. P. and Debevere J. 2009. Reducing salt intake from meat products by combined use of lactate and diacetate salts without affecting microbial stability. Int. J. Food Sci. Tech. 44: 337-341.
- Dezfulian C., Shiva S., Alekseyenko A., Pendyal A., Beiser D.G., Munasinghe J.P., Anderson S.A., Chesley C.F. et al. 2009. Nitrite Therapy after Cardiac Arrest Reduces Reactive Oxygen Species Generation, Improves Cardiac and

- Neurological Function, and Enhances Survival *via* Reversible Inhibition of Mitochondrial Complex I. *Circulation*. 120: 897-905.
- Directive. 2006. Directive 2006/52/EC of the European Parliament and of the Council of 5 July 2006 amending directive 95/2/EC on food additives other than colours and sweeteners and Directive 95/35/EC on sweeteners for use in foodstuffs. O. J. L204 of 26.7.2006.
- Doerr R. C., Fox J. B., Lakritz. and Fiddler W. 1981. Determination of nitrite in cured meats by chemiluminiscence. *Anal. Chem.* 53: 381-384.
- Doores S. 2005. Organic Acids. Antimicrobials in Food. Davidson P. M., Sofos J. N. and Branen A. L (Eds.). Florida, Taylor & Francis Group. Page 101.
- Dordevic V., Vuksan B., Radetic P., Durdica H. and Mitkovic M. 1980. Prilog ispitivanju uticaja pojedinih faktora na promene sadrzaja nitrita u mesu. *Tehnologija mesa*. 21: 287-290.
- Dorsa W. J., Cutter C. N. And Siragusa G. R. 1997. Effect of acetic acid, lactic acid and trisodium phosphate on the microflora of refrigerated beef carcass surface tissue inoculated with *Escherichia coli* O157:H7, *Listeria innocua* and *Clostridium sporogenes*. *J. Food Prot.* 60: 619-624.
- Duffy L. L., Vanderlinde P. B. and Grau F. H. 1994. Growth of *Listeria monocytogenes* on vacuum packed cooked meats: effect of pH, aw, nitrites and ascorbates. *Int. J. Food Microb.* 23: 377-390.
- Dykhuizen R. S., Frazer R., Duncan C., Smith C. C., Golden M., Benjamin N. and Leifert C. 1996. Antimicrobial effect of acidified nitrite on gut pathogens: importance of dietary nitrate in host defense. *Antimicrob. Agents and Chemoth.* 40: 1422-1425.
- Eggers N. J. and Cattle D. L. 1986. High performance liquid chromatography for the determination of nitrate and nitrite in cured meat. *J. Chrom.* 354: 490-494.
- Ellebracht E. A., Castillo A., Lucia L. M., Miller R. K. and Acuff G. R. 1999. Reduction of pathogens using hot water and lactic acid on beef trimmings. *J. Food Sci.* 64: 1094-1099.
- FDA. 2011. Alphabetical list of SCOGS substances. Available online at <http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASSubstancesSCOGSDatabase/ucm084104.htm>. Accessed on July 29th, 2011.
- Fiddler W., Pensabene J. W., Piotrowski E. G., Phillips J. G., Keating J., Mergens W. J. et al. 1978. Inhibition of formation of volatile nitrosamines in fried bacon by the use of cure-solubilized alfa-tocopherol. *J. Agr. Food Chem.* 26: 653-656.
- Fine D. H., Ross R., Rounbehler D. P., Silvergleid A. and Song L. 1977. Formation in vivo of volatile N-nitrosamines in man after ingestion of cooked bacon and spinach. *Nature*. 265: 753-755.
- Food Standards Agency. 2002. McCance & Widdowson's The composition of Foods (6th ed.). United Kingdom, Cambridge.

- Forte J. G., Miguel J. M., Miguel M. J., de Padua F. and Rose G. 1989. Salt and blood pressure: A community trial. *J. Hum Hypertens.* 3: 179-184.
- Fox J. B. and Nicholas R. A. 1974. Nitrite in Meat. Effect of various compounds on Loss of nitrite. *J. Agr. Food Chem.* 22: 302-306.
- Fox J. B., Doerr R. C. and Gates R. 1984. Effect of residual ascorbate on determination of nitrite in commercial cured meat products. *J. Assoc. Off. Anal. Chem.* 67: 692-697.
- Frederick T. L., Miller M. F., Thompson L. D. and Ramsey C. B. 1994. Microbiological properties of pork cheek meat as affected by acetic acid and temperature. *J. Food Sci.* 59: 300-305.
- Frye C. B., Hand L. W., Calkins C. R. and Mandigo R. W. 1986. Reduction or replacement of sodium chloride in a tumbled ham product. *J. Food Sci.* 51: 836-837.
- Funazo K., Tanaka M. and Shono T. 1980. Determination of nitrite at parts-per-billion levels by derivatization and electron capture gas chromatography. *Anal. Chem.* 52: 1222-1224.
- Gershon H. and Shanks L. 1979. Antifungal activity of fatty acids and derivatives: Structure activity relationships. *The Pharmacological effects of lipids.* Kabara J. J. (Ed.). Champaign, American Oil Chemist's Society. Pages 51-62.
- Gibson A. M., Roberts T. A. and Robinson A. 1984. Factors controlling the growth of *Clostridium botulinum* types A and B in pasteurized cured meats VI. Nitrite monitoring during storage of pasteurized pork slurries. *J. Food Technol.* 19: 29-44.
- Gomis D. B. 1992. HPLC analysis of organic acids. *Food Analysis by HPLC.* Nollet L (Ed.). M. New York, Marcel Decker. Page 371.
- Gough B. J. and Alford J. A. 1965. Effect of curing agents on the growth and survival of food- poisoning strains of *Clostridium perfringens*. *J. Food Sci.* 30: 1025-1028.
- Grau F. H. and Vanderlinde P. B. 1992. Occurrence, numbers and growth of *Listeria monocytogenes* on some vacuum-packaged processed meats. *J. Food Prot.* 55: 4-7.
- Greer G. G. and Dilts D. D. 1995. Lactic acid inhibition of the growth of spoilage bacteria and cold tolerant pathogens on pork. *Int. J. Food Microb.* 25: 141-151.
- Gry, J., Damm Rasmussen, N.J., Klinth Jensen, W., Brandt, I.G. and Fabech, B. 1983. Investigations on effects of nitrite in commercially prepared Danish cured meat products. A report of The Federation of Danish Pig Producers and Slaughterhouses and the National Food Agency of Denmark.
- Hamm R. 1986. Functional properties of the myofibrillar system. *Muscle as food.* Bechtel P. J (Ed.). New York, Academic Press. Pages 135-200.
- Hayes H. and Britton H. 1936. Process for the production of sodium nitrite. US Patent, 2032699. Serial Number 64,200.

- He F. J. and McGregor G. A. 2002. Effect of modest salt reduction on blood pressure: a meta-analysis of randomized trials. Implications for public health. *J. Hum. Hypertens.* 16: 761-770.
- He F. J. and McGregor G. A. 2006. Effect of long-term modest salt reduction on blood pressure (review). The Cochrane Collaboration. New Jersey, Wiley Publishers.
- He F. J. and McGregor G. A. 2008. A comprehensive review on salt and health and current experience of worldwide salt reduction programmes. *J. Human Hypert.* 1-22.
- Hill, L.H., Webb, N.B., Mongol, L.D. and Adams, A.T. 1973. Changes in residual nitrite in sausages and luncheon meat products during storage. *J. Milk Food Technol.* 36: 515-519.
- Hoffman P. and Possin I. 2000. Adding organic acids to high moisture corn. Focus on forage. Available online at <http://www.uwex.edu/ces/crops/uwforage/HMC-OA.pdf>. Accessed on July 29th, 2011.
- Honikel K. 2008. The use and control of nitrate and nitrite for the processing of meat products. *Meat Sci.* 78: 68-76.
- Hord N. G., Tang Y. and Bryan N. S. 2009. Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *Am. J. Clin. Nutr.* 1: 1-10.
- Houtsma P. C., de Wit J. C. and Rombouts F. M. 1993. Minimum inhibitory concentration (MIC) of sodium lactate for pathogens and spoilage organisms occurring in meat products. *Int. J. Food Microb.* 20: 247-257.
- Hsu J., Arcot J. and Lee N. A. 2009. Nitrate and nitrite quantification from cured meat and vegetables and their estimated dietary intake in Australians. *Food Chem.* 115: 334-339.
- Hunt J. and Seymour D. J. 1985. Method for measuring nitrate-nitrogen in vegetables using anion-exchange high performance liquid chromatography. *J. Chrom.* 295: 471-478.
- ICMSF. 1996. *Clostridium perfringens*. Microorganisms in Food. Roberts T (Ed.). London, UK, Blackie Academic and Professional. Pages 112-125.
- Jackson A. I., Sullivan G. A., Kulchaiyawat C., Sebranek J. G. and Dickson J. 2011. Survival and growth of *Clostridium perfringens* in commercial no-nitrate-or-nitrite-added (natural and organic) frankfurters, hams, and bacon. *J. Food Prot.* 74: 410-416.
- Jackson A., Kulchaiyawat C., Sullivan G. A., Sebranek J. G. and Dickson J. 2011. Use of natural ingredients to control growth of *Clostridium perfringens* in naturally cooked frankfurters and hams. *J. Food Prot.* 74: 417-424.
- Jackson P. E., Haddad P.R. and Dilli S. 1984. Determination of nitrate and nitrite in cured meats using high-performance liquid chromatography. *J. Chrom.* 295: 471-478.
- Jacoby C. H. 1972. Salt solution mining and geothermal heat utilization system. US Patent No 3,676,078. Serial No 21,051.

- Jensen J. M., Robbins K. L., Ryan K. J., Homco-Ryan C., McKeith F K. and Brewer M. S. 2003. Effect of lactic and acetic acid salts on quality characteristics of enhanced pork during retail display. *Meat Science*. 63: 501-508.
- Jimenez-Colmenero F. and Blazquez Solana J. 2009. Handbook of processed meats and poultry analysis. Nollet M. and Toldra F (Eds.). Florida, Taylor and Francis Group. Page 100.
- Jo C., Ahn H. J., Son J. H. Lee J. W. and Byun M. W. 2003. Packaging and irradiation effect on lipid oxidation, color, residual nitrite content and nitrosamine formation in cured pork sausage. *Food Control*. 14: 7-12.
- Juneja V. K. 2003. Predictive model for the combined effect of temperature, sodium lactate and sodium diacetate on the heat resistance of *Listeria monocytogenes* in beef. *J. Food Prot.* 66: 804-811.
- Juneja V. K. and Marks H. M. 2002. Predictive model for growth of *Clostridium perfringens* during cooling of cooked cured chicken. *Food Microb.* 19: 313-327.
- Juneja V. K. and Marmer B. S. 1996. Growth of *Clostridium perfringens* from spore inocula in *sous-vide* turkey products. *Int. J. Food Microb.* 32: 115-123.
- Juneja V. K. and Thippareddi H. 2004a. Control of *Clostridium perfringens* in a model roast beef by salts of organic acids during chilling. *J. Food Safety*. 24: 95-108.
- Juneja V. K. and Thippareddi H. 2004b. Inhibitory effects of organic acid salts on growth of *Clostridium perfringens* from spore inocula during chilling of marinated ground turkey breast. *Int. J. Food Microb.* 93: 155-163.
- Juneja V. K., Huang L. and Thippareddi H. 2006. Predictive model for growth of *Clostridium perfringens* in cooked cured pork. *Int. J. Food Microb.* 110: 85-92.
- Juneja V. K., Novak J. S., Marks M. and Gombas D. E. 2001. Growth of *Clostridium perfringens* from spore inocula in cooked cured beef: development of a predictive model. *Innov. Food Sci. and Emerg. Techn.* 2: 289-301. 32: 115-123.
- Kang D. H., Koohmaraie M. and Siragusa G. R. 2001. Application of multiple interventions for microbial decontamination of beef trim. *J. Food Prot.* 64: 63-71.
- Katan M. B. 2009. Nitrite in foods: harmful or healthy? *Am. J. Clin. Nutr.* 90: 11-12
- Keeton J. T., Osburn W. N., Hardin M. D., Bryan N. S. and Longnecker M. T. A national survey of the nitrate/nitrite concentrations in cured meat products and non-meat foods available at retail. Executive summary. National Pork Board Project # 08-124.
- Kilic B., Cassens R. G. and Borchert L. L. 2001. Influence of turkey meat on residual nitrite in cured meat products. *J. Food Prot.* 64: 235-239.
- Knight D. 1992. Humphry Davy: Science and power. United Kingdom, Cambridge University Press. Page 77.
- Knight T. M., Forman D., Al-Dabbagh S. A. and Doll R. 1987. Estimation of dietary intake of nitrate and nitrite in Great Britain. *Food Chem. Toxicol.* 25: 277-285.

- Kramlich W. E., Pearson. A. M. and Tauber S. W. 1973. Processed Meats. Connecticut, AVI Publishing Company.
- Krebs H. A., Wiggins D., Stubs M., Sols A. and Bedoya F. 1983. Studies on the mechanism of the antifungal action of benzoate. *Biochemical Journal*. 214: 657-663.
- Kumar D., Branch B. G., Pattillo C. B., Hood J., Thoma S., Simpson S. et al. 2008. Chronic sodium nitrite therapy augments ischemia-induced angiogenesis and arteriogenesis. *PNAS*. 105: 7540-7545.
- Kurlansky M. 2002. Salt, A world history. New York, Walker Publishing Co.
- Labbe R. G. 2000. *Clostridium perfringens*. The microbiological safety and quality of food, Vol 2. B.M. Lund, T.C. Baird-Parker, and G.W. Gould (Eds.). Gaithersburg, MD, Aspen Publishers Inc. Pages 1110-1135.
- Labbe R. G. and Duncan C. L. 1970. Growth from spores of *Clostridium perfringens* in the presence of sodium nitrite. *Appl. Microb.* 19: 353-359.
- Labbe R.G. and Huang T. 1995. Generation times and modeling of enterotoxin-positive and enterotoxin-negative strains of *Clostridium perfringens* in laboratory media and ground beef. *J. Food Prot.* 58: 1303-1306.
- Labbe R.G. and Juneja V. 2006. Foodborne infections and intoxications (3 Ed). Riemann H. and Clier D (Eds.). California, Academic Press. Page 137.
- Larson J. R., Tingstad J. E. and Swadesh J. K. 2002. HPLC: Practical and industrial applications (2nd ed.). Swadesh J. K (Ed.). Florida, CRC Press. Page 11.
- Law M. R., Frost C.D. and Wald N. J. 1991. Analysis of data from trials of salt reduction. *BMJ*. 302: 819-823.
- Ledermann W. 2007. Una historia personal de las bacterias. Santiago, RIL Editores. Pages 103-104.
- Li J. and McClane B. 2006. Comparative effects of osmotic, sodium nitrite induced, and pH induced stress on growth and survival of *Clostridium perfringens* Type A isolates carrying chromosomal or plasmid-borne enterotoxin genes. *Appl. And Env. Microb.* 72: 7620-7625.
- Lin G. C., Mittal G. S. and Barbut S. 1991. Optimization of tumbling and KCl substitution in low sodium restructured hams. *J. Muscle Foods*. 2: 71-91.
- Lin H., Sebranek J. G., Galloway D. and Lind K. 1980. Effect of sodium erythorbate and packaging conditions of color stability of sliced bologna. *J. Food Sc.* 45: 115-118.
- Lin P.Y., Whang L.M., Wu Y.R., Ren W.J., Hsiao C.J., Li S.L. and Chang J.S. 2007. Biological hydrogen production of the genus *Clostridium*: Metabolic study and mathematical model simulation. *Int. J. Hyd. Energy*. 32: 1728-1735.
- Lindstrom M., Heikinheimo A, Lahti P. and Korkeala H. 2011. Novel insights into the epidemiology of *Clostridium perfringens* type A poisoning. *Food Microbiology*. 28: 192-198.

- Lohmeier-Vogel E. M., Sopher C. R. and Lee H. 1998. Intracellular acidification as a mechanism for the inhibition by acid hydrolysis-derived inhibitors of xylose fermentation by yeast. *J. Ind. Microb. Biotech.* 20: 75-81.
- Low salt products-Directory. Available on line at http://www.lowsaltfoods.com/food_center/lsfoods/dir_lsfoods.htm. Accessed on July 8th, 2011.
- Lueck E. 1980. *Antimicrobial Food Additives: characteristics, uses, effects*. New York, Springer-Verlag.
- Maas M. R., Glass K. A. and Doyle M. P. 1989. Sodium lactate delays toxin production by *Clostridium botulinum* in cook-in-bag turkey products. *Appl. Environ. Microb.* 55: 22-26.
- Mackay J. and Mensah G. 2004. *The Atlas of Heart Disease and Stroke*. United Kingdom, Myriad Editions.
- Marz U. 2002. World markets for citric, ascorbic, isoascorbic acids: Highlighting antioxidants in food. Food and Beverage. London, BCC., Inc.
- Massey R. C. 1996. Nitrates and nitrites in Food and Water. Hill M. J (Ed.). Cambridge, Woodhead Publishing. Pages 13-32.
- Mbandi E. And Shelef L. A. 2001. Enhanced inhibition of *Listeria monocytogenes* and *Salmonella enteritidis* in meat by combinations of sodium lactate and diacetate. *J. Food Prot.* 64: 640-644.
- McClure P. 2002. *Foodborne pathogens: hazards, risks analysis and control*. Cambridge, Woodhead Publishing. Page 423.
- McColl J. G. And Polhman A. A. 1986. Soluble organic acids and their chelating influence on Al and other metal dissolutions from forest soil. *Water, air and soil pollution.* 31: 917-927.
- McMindes M. K. and Siedler A. J. 1988. Nitrite mode of action: inhibition of yeast pyruvate decarboxylase (E.C.4.1.1.1.) and clostridial pyruvate:ferredoxin oxidoreductase (E.C.1.2.7.1) by nitric oxide. *J. Food Sci.* 53: 919-919, 931.
- Merusi C., Corradini C., Cavazza A., Borromei C. and Salvadeo P. 2010. Determination of nitrates, nitrites and oxalates in food products by capillary electrophoresis with pH-dependent electroosmotic flow reversal. *Food Chem.* 120: 615-620.
- Miller R. K. and Acuff G. R. 1994. Sodium lactate effects pathogens in cooked beef. *J. Food Sci.* 59: 15-19.
- Miwa N., Nishina T., Kubo S., Atsuno M. and Honda H. 1998. Amount of enterotoxigenic *C. perfringens* in meat detected by nested PCR. *Int. J. of Food Microb.* 42:195-200.
- Mohammed A. A., Mubarak A. T., Fawy K. F and El-Shahat M. F. 2008. Modification of AOAC method 973.31 for determination of nitrite in cured meats. *J. AOAC Int.* 91: 820-827.

- Moller J., Jensen J., Olsen M., Skibsted L. and Bertelsen G. 2000. Effect of residual oxygen on colour stability during chill storage of sliced, pasteurized ham packaged in modified atmosphere. *Meat Science*. 54: 399-405.
- Monahan F. J. and Troy D. J. 1997. Overcoming sensory problems in low fat and low salt products. *Advances in Meat Research. Production and Processing of Healthy Meat, Poultry and Fish Products*. Pearson A. M. and Dutson T. R (Eds.). London, Blackie Academic and Professional. Page 257.
- Montville T. and Matthews K. 2008. *Food Microbiology: An introduction* (2 ed). Washington D.C., ASM Press. Pages 221-222.
- Moran D. M., Tannembaum S. R. and Archer M. C. 1975. Inhibitor of *Clostridium perfringens* formed by heating sodium nitrite in a chemically defined medium. *Appl. Microb.* 30: 838-843.
- Motala C. and Steinman H. 2008. Food allergy, preservatives and asthma. Available online at <http://www.asthma.co.za/articles/ref13.htm>. Accessed on July 29th, 2011.
- Murry A. C., Hinton A. and Morrison H. 2004. Inhibition of growth of *Escherichia coli*, *Salmonella Typhimurium* and *Clostridium perfringens* on chicken feed media by *Lactobacillus salivarius* and *Lactobacillus platarum*. *Int. J. Poultry Sci.* 3: 603-607.
- O'Daly A. 2001. *The elements: Sodium*. New York, Marshall Cavendish Corporation. Page 11.
- O'leary V. and Solberg M. 1976. Effect of sodium nitrite inhibition on intracellular thiol groups and on the activity of certain glycolytic enzymes in *Clostridium perfringens*. *Appl. and Env. Microb.* 31: 208-212.
- Olsman W. J. and van Leeuwen C. M. 1977. The assay of protein-bound nitrite in meat products. *Zeitschrift fur Lebensmittel Untersuchung und Forschung*. 164: 239-242.
- Osgood M. and Ocorr C. 2009. *The ultimate absolute guide to Lehninger Principles of Biochemistry Study Guide and Solution manual*. New York, W. H. Freeman and Company.
- Oztekin N., Said Nutku M. and Bedia Erim F. 2002. Simultaneous determination of nitrite and nitrate in meat products and vegetables by capillary electrophoresis. *Food Chem.* 76: 103-106.
- Paulsen P. and Smulders F. J. 2003. Combining natural antimicrobial systems with other preservation techniques: the case of meat. *Food preservation techniques*. Zeuthen P. and Sorensen L. B (Eds.). Cambridge, CRC Press. Page 75.
- Pegg R. and Shahidi F. 2000. *Nitrite curing of meat: the N-Nitrosamine problem and nitrite alternatives*. Connecticut, Food and Nutrition Press.
- Permpasert J. and Devahastin S. 2005. Evaluation of the effects of some additives and pH on surface tension of aqueous solutions using a drop-weight method. *J. Food Eng.* 70: 219-226.

- Perry I.J. and Beevers D. G. 1992. Salt intake and stroke: a possible direct effect. *J. Hum Hypertens.* 6: 23-25.
- Pietinen P., Valsta L. M., Hirvonen T. and Sinkko H. 2008. Labelling the salt content in foods: a useful tool in reducing sodium intake in Finland. *Public Health Nutr.* 11: 335-340.
- Piper P. W. 1999. Yeast superoxide dismutase mutants reveal a pro-oxidant action of weak organic acid food preservatives. *Free Radical Biology and Medicine.* 27: 11-12.
- Piper P., Calderon C. O., Hatzixanthis K. and Mollapour M. 2001. Weak acid adaptation: The stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiology.* 147: 2635-2642.
- Plumridge A., Hesse S. J., Watson A. J., Lowe K. C., Stratford M. and Archer D. B. 2004. The weak acid preservative sorbic acid inhibits conidial germination and mycelial growth of *Aspergillus niger* through intracellular acidification. *Appl. Env. Microb.* 70: 3506-3511.
- Prasad S. and Avinesh A. 2008. Nitrate-N determination in leafy vegetables: Study of the effects of cooking and freezing. *Food Chem.* 106: 772-780.
- Reddy D., Lancaster J. R. and Cornforth D. P. 1983. Nitrite inhibition of *Clostridium botulinum*: electron spin resonance detection of iron-nitric oxide complexes. *Science.* 221: 769-770.
- Reece P. and Hird H. 2000. Modification of the ion exchange HPLC procedure for the detection of nitrate and nitrite in dairy products. *Food Addit. and Contam.* 17: 219-222.
- Ricke S. C. 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poult. Sci.* 82: 632-639.
- Riera J. B., Martinez M. R., Salcedo R. C., Juncosa G. M. and Sellart J. C. 1996. Process for producing a low sodium meat product. US Patent 5534279.
- Rincon F., Martinez B. and Delgado J. M. 2003. Detection of factors influencing nitrite determination in meat. *Meat Scie.* 65: 1421-1427.
- Roberts T. A. and Dainty R. H. 1996. Nitrates and nitrites in Food and Water. Hill M. J (Ed.). Cambridge, Woodhead Publishing. Pages 117-130.
- Robinson A., Gibson A. M. and Roberts T. A. 1982 Factors controlling the growth of *Clostridium botulinum* types A and B in pasteurized meats. V. Prediction of toxin production: non-linear effects of storage temperature and salt concentration. *J. Food Technol.* 17: 727-744.
- Rose B.D. and Post T.W. 2001. Clinical physiology of acid-based and electrolyte disorders. United States, McGraw-Hill. Pages 746-794.
- Rounds M. A. and Gregory J. F. 2003. Food Analysis (3rd ed.). Nielsen S. S (Ed.). New York, Kluwer Academic/Plenum Publishers. Page 461.

- Rounds M. A. and Nielsen S. S. 2003. Food Analysis (3rd ed.). Nielsen S. S (Ed.). New York, Kluwer Academic/Plenum Publishers. Page 438.
- Ruusunen M. and Puolanne E. 2005. Reducing sodium intake from meat products. Meat Sci. 70: 531-541.
- Ruusunen M., Simolin M. and Puolanne E. 2001. The effect of fat content and flavor enhancers on the perceived saltiness of cooked bologna-type sausages. J. Muscle Foods. 12: 107-120.
- Ryan K. and Ray C. 2010. Sherris Medical Microbiology (5ed). New York, McGraw Hill. Pages 520-521.
- Sabah J. R., Juneja V. K. and Fung D. Y. 2004. Effect of spices and organic acids on the growth of *Clostridium perfringens* during cooling of cooked ground beef. J. Food Prot. 67: 1840-1847.
- Sabah J., Thippareddi H., Marsden J.L. and Fung D. 2003. Use of organic acids for the control of *Clostridium perfringens* in cooked vacuum-packaged restructured roast beef during an alternative cooling procedure. J. Food Prot. 66:1408-1412.
- Salt Institute. 2011. Salt in history. Available at <http://www.saltinstitute.org/Uses-benefits/Salt-in-history>. Accessed on July 6th, 2011.
- Sanchez-Plata M. X., Amezcuita A., Blankenship E., Burson D., Juneja V. K. and Thippareddi H. 2005. Predictive model for *Clostridium perfringens* growth in roast beef during cooling and inhibition of spore germination and outgrowth by organic acid salts. J. Food Prot. 68: 2594-2605.
- Sanchez-Plata, M. X. 2004. Germination and outgrowth of *Clostridium perfringens* spores during chilling of thermally processed ready-to-eat meat products. Ph.D. dissertation, University of Nebraska, Lincoln.
- Santamaria P. 2006. Nitrate in vegetables: toxicity, content, intake and EC regulation. J. Sci. Food Agr. 86: 10-17.
- Sarker M., Shivers R., Sparks S., Juneja V. and McClane B. 2000. Comparative experiments to assess the effect of heating on vegetative cells and spores of *Clostridium perfringens* isolates carrying plasmid genes versus chromosomal enterotoxin genes. Appl. Env. Microb. 66: 3234-3240.
- Sarraga C., Gil M., Arnau J., Monfort J. and Cusso R. 1989. Effect of curing salt and phosphate on the activity of porcine muscle proteases. Meat Sci. 25:241-249.
- Scallan E., Hoekstra R., Angulo F., Tauxe R., Widdowson M., Roy S., Jones J. and Griffin P. 2011. Foodborne illness acquired in the United States-Major Pathogens. Emerg. Infect. Dis. 17: 7-14.
- Schuddeboom L. J. 1993. Nitrates and nitrites in foodstuffs. Council of Europe Press, Publishing and Documentation Service, ISBN 92-871-2424-6.
- Scientific panel on food additives, flavourings, processing aids and materials in contact with food. 2003. Opinion of the scientific panel on biological hazards on the request from the commission. The effects of nitrites/nitrates on the microbiological safety of meat products. EFSA Journal. 14: 1-31.

- Sebranek J. and Fox J. 1985. A review of nitrite and chloride chemistry: Interactions and implications for cured meats. *J. Sci. Food Agr.* 36:1169-1182.
- Seifter J., Ratner A. and Sloane D. 2005. *Concepts in Medical Physiology*. Philadelphia, Lippincott, Williams and Wilkins.
- Sen N. and Baddoo P. 1997. Trends in the levels of residual nitrite in Canadian cured meat products over the past 25 years. *J. Agric. Food Chem.* 45: 4714-4718.
- Shelef L. A. 1994. Antimicrobial effects of lactates: A review. *J. Food Prot.* 57: 445-450.
- Sherwood L. 2010. *Human physiology: from cells to systems*. California, Brooks/Cole Cengage Learning. Page 66.
- Shimizu T., Ohtani K., Hirakawa H., Ohshima K., Yamashita A., Shiba T., Ogasawara N., Hattori M., Kuhara S. and Hayashi H. 2001. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. *PNAS*. 99: 996-1001.
- Sigrid B. and Granum P. 2002. *Clostridium perfringens* and foodborne infections. *Int. J. Food Mic.* 74: 195-202.
- Slack P. T. 1987. *Analytical methods manual*. Leatherhead Food R. A.
- Smelt J. P., Raatjes J. G., Crowther J. S. and Verrips C. T. 1982. Growth and toxin formation by *Clostridium* at low pH values. *J. Appl. Bact.* 52: 75-82.
- Smulders F. J. and Greer G. G. 1998. Integrating microbial decontamination with organic acids in HACCP programmes for muscle foods: Prospects and controversies. *Int. J. Food Microb.* 44: 149-169.
- Smulders F. J., Barendsen P., Van Logtestijn J. G., Mossel D. A. and Van der Marel G. M. 1986. Review: Lactic acid: considerations in favor of its acceptance as a meat decontaminant. *Int. J. Food Sci. Technol.* 21: 419-436.
- Sofos J. N., Busta F. F., Bothipaksa K., Allen C. E., Robach M. C. and Paquett W. 1980. Effect of various concentrations of sodium nitrite and potassium sorbate on *Clostridium botulinum* toxin production in commercially prepared bacon. *J. Food Sci.* 45: 1285-1292.
- Solberg M. and Elking B. 1970. Effect of processing and storage conditions on the microflora of *Clostridium perfringens*-inoculated frankfurters. *J. Food Sci.* 35: 126-129.
- Songer J. and Post K.W. 2005. *Veterinary Microbiology: Bacterial and Fungal Agents of Animal Disease*. St. Louis, Mo., Elsevier Saunders. Pages 320-325.
- Sperber W. H. 1983. Influence of water activity on foodborne bacteria – A review. *J. Food Prot.* 46: 142-150.
- Staack N., Arhne L., Borch E. and Knorr D. 2008. Effect of temperature, pH and controlled water activity on inactivation of spores of *Bacillus cereus* in paprika powder by near-IR radiation. *J. Food Engg.* 89: 319-324.
- Stagnitta P., Micalizzi B. and Steffani de Guzman A. 2002. Prevalence of enterotoxigenic *C. perfringens* in meats in San Luis, Argentina. *Anaerobe.* 8: 253-258.

- Stekelenburg F. K. and Kant-Muermans M. L. 2001. Effect of sodium lactate and other additives in a cooked ham product on sensory quality and development of a strain of *Lactobacillus curvatus* and *Listeria monocytogenes*. Int. J. Food Microb. 66: 197-203.
- Strain H. H. and Sherma J. 1967. Michael Tswett's contributions to 60 years of chromatography. J. Chem. Educ. 44: 235.
- Stratford M. and Eklund T. 2003. Organic acids and esters. Food preservatives (2nd ed). Russell N. J. and Gould G. W (Eds.). New York, Kluwer Academic. Pages 48-76.
- Strazzullo P., D'Elia L., Kandala N. B., and Cappuccio F. P. 2009. Salt intake, stroke, and cardiovascular disease: meta-analysis of prospective studies. BMJ. 339: b4567.
- Stringer S. C. and Pin C. 2005. Microbial risks associated with salt reduction in certain foods and alternative options for preservation. Technical report. United Kingdom, Institute of Food Research.
- Suomalainen H. and Oura E. 1955. Buffer effect in fermentation solutions. Exp. Cell Research. 9: 355-359.
- Taormina, P.J., Bartholomew, GW and Dorsa, WJ. 2003. Incidence of *Clostridium perfringens* in commercially produced cured raw meat product mixtures and behavior in cooked products during chilling and refrigerated storage. J. Food Prot. 66: 72-81.
- Tapia M. S., Alzamora S. M. and Chirife J. 2007. Effects of water activity (A_w) on microbial stability: As a hurdle in food preservation. Water activity in foods: fundamentals and applications. Barbosa-Canovas G. B (Ed.). Iowa, Blackwell Publishing and the Institute of Food Technologists. Pages 253-265.
- Taylor R. S., Ashton K. E., Moxham T., Hooper L. and Ebrahim S. 2011. Reduced dietary salt for the prevention of cardiovascular disease: A meta-analysis of randomized control trials (Cochrane Review). Am. J. Hypertens. 24: 843-853.
- Terrel R. N. 1983. Reducing the sodium content of processed meats. Food Technol. 37: 66-71.
- Theron M. M. and Rykers J. F. 2011. Organic acids and food preservation. Boca Raton, Florida, CRC Press.
- Thippareddi H., Juneja V.K., Phebus R., Marsden J.L. and Kastner C.L. 2003. Control of *Clostridium perfringens* germination and outgrowth by buffered sodium citrate during chilling of roast beef and injected pork. J. Food Prot. 66:376-381.
- Thomson B. M., Nokes C. J. and Cressey P. J. 2007. Intake and risk assessment of nitrate and nitrite from New Zealand foods and drinking water. Food Add. Cont. 24: 113-121.
- Tompkin R. B. 2005. Antimicrobials in Food. Davidson P. M., Sofos J. N. and Branen A (Eds.). L. Florida, Taylor & Francis Group. Page 170-171.
- Tompkin R. B., Christiansen L. N. and Shaparis A. B. 1978. Enhancing nitrite inhibition of *Clostridium botulinum* with isoascorbate in perishable canned cured meat. Appl. Env. Microb. 35: 59-61

- Tompkin R. B., Christiansen L. N. and Shaparis A. B. 1979. Iron and the antitoxigenic efficacy of nitrite. *Appl. Env. Microb.* 37: 351-353.
- Tsikis D. 2007. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction in the L-arginine/nitric oxide area of research. *J. Chrom. B.* 851: 51-70.
- Tuomilehto J., Jousilahti P., Rastenyte D., Moltchanov V., Tanskanen A., Pietinen P. et al. 2001. Urinary sodium excretion and cardiovascular mortality in Finland: A prospective study. *Lancet.* 357: 848-851.
- USDA. 2005. Dietary Guidelines for Americans (7th ed). Washington D. C., US Government Printing Office. Page 40.
- USDA. 2005. Food nutrient database. Available online at <http://www.nal.usda.gov/fnic/foodcomp/search/>. Accessed on July 20th, 2011.
- USDA-FSIS. 1999. Performance standards for the production of certain meat and poultry products. FSIS Directive 7111.1 Federal Register 64:732-749.
- USDA-FSIS. 2000. Food additives for use in meat and poultry products: sodium diacetate, sodium acetate, sodium lactate and potassium lactate. Code of Federal Regulations, 9 CFR Part 424 65:3121-3123.
- Usher C. D. and Telling G. M. 1975. Analysis of nitrate and nitrite in foodstuffs: a critical review. *J. Sci. Food Agric.* 26: 1793-1805.
- Valenzuela-Martinez C., Pena-Ramos A., Juneja V. K., Korasapati N. R., Burson D. and Thippareddi H. 2010. Inhibition of *Clostridium perfringens* spore germination and outgrowth by buffered vinegar and lemon juice concentrate during chilling of ground turkey roast containing minimal ingredients. *J. Food Prot.* 73: 470-476.
- Valli M., Sauer M., Branduardi P., Borth N., Porro D. and Mattanovich D. 2006. Improvement of lactic acid production in *Saccharomyces cerevisiae* by cell sorting for high intracellular pH. *Appl. Env. Microb.* 72: 5492-5499.
- van Netten P., Mossel D. A. and Huls J. 1995. Lactic acid decontamination of fresh pork carcasses: A pilot plant study. *Int. J. Food Microb.* 25: 1-9.
- Vasseur C., Baverel L., Hebraud M. and Labadie J. 1999. Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes*. *J. Appl. Microb.* 86: 469-476.
- Velugoti P.R., Bohra L. K., Juneja V. J. and Thippareddi H. 2007. Inhibition of germination and outgrowth of *Clostridium perfringens* by lactic acid salts during cooling of injected turkey. *J. Food Prot.* 70: 923-929.
- Wellings D. A. 2006. A practical handbook of preparative HPLC. Amsterdam, Elsevier. Pages 74-75.
- WHO. 2007. Nitrate and nitrite in drinking water. Background document for development of WHO guidelines for drinking-water quality. Switzerland, WHO Press.
- Witschi H. 2002. Profiles in Toxicology: John Morrison Barnes 1913-1975. *Toxicological Sciences.* 68: 267-269.

- Wootton M., Kok S. H. and Buckle K. A. 1985. Determination of nitrite and nitrate levels in meat and vegetable products by high performance liquid chromatography. J. Sci. Food Agric. 36: 297-304.
- Yarbrough J. M., Rake. J. B. and Eagon R. G. 1980. Bacterial inhibitory effects of nitrite: inhibition of active transport, but not of group translocation and of intracellular enzymes. Appl. and Env. Microb. 39: 830-834.
- Zaika L. L. 2003. Influence of NaCl content and cooling rate on outgrowth of *Clostridium perfringens* spores in cooked ham and beef. J. Food Prot. 66: 1599-1603.
- Zanardi E., Dazzi G., Madarena G. and Chizzolini R. 2002. Comparative study on nitrite and nitrate ions determination. Ann. Fac. Medic. Vet. di Parma. 22: 79-86.
- Zhang Y.Z., Jin B. and Kelly J. M. 2007. Production of lactic acid from renewable materials by *Rhizopus fungi*. Bioch. Eng. J. 35: 251-263.
- Zumft W. 1997. Cell biology and molecular basis of denitrification. Microb. Mol. Biol. Rev. 61:533-616.

CHAPTER 2

7. Effect of Sodium Nitrite and Sodium Erythorbate on Germination and Outgrowth of *Clostridium perfringens* Spores in Ham During Abusive Cooling

7.1 ABSTRACT

The effect of sodium nitrite and sodium erythorbate on the germination and outgrowth of *Clostridium perfringens* in ham during abusive cooling was evaluated. Ham was formulated with ground pork, NaNO₂ (0, 50, 100, 150 or 200 ppm) and/or sodium erythorbate (0 or 547 ppm). A portion of the meat (10 g) was transferred to a vacuum bag and inoculated with a three-strain *C. perfringens* spore cocktail to obtain a spore population of ca. 2.5 log spores/g. Samples were vacuum packaged, heat treated (75°C, 20 min) and cooled within 15 h from 54.4 to 4.4°C after 3 or 24 h after preparation. Residual nitrite concentration in the product was determined before and after heat treatment using High Performance Liquid Chromatography (HPLC). *C. perfringens* population increases of 1.46 and 4.20 log CFU/g were observed in ham (control) processed after 3 and 24 h of preparation, respectively and exponentially cooled within 15 h. Incorporation of NaNO₂ in the ham formulation resulted in inhibition of *C. perfringens* spore germination and outgrowth with greater inhibition was observed with higher concentrations of sodium nitrite. Addition of sodium erythorbate resulted in greater *C. perfringens* populations subsequent to 15 h of abusive cooling. Residual nitrite concentrations were similar under the experimental conditions used in this study. Inhibition of *C. perfringens* spore germination and outgrowth when the product was heat treated and cooled immediately after preparation could be explained as a function of the presence of residual oxygen in the meat.

Key words: sodium nitrite, sodium erythorbate, residual nitrite, *Clostridium perfringens*

7.2 Introduction

Clostridium perfringens is a Gram positive, large, non-motile rod. It is an encapsulated microorganism with the capacity to form spores that are highly resistant to environmental conditions (Ryan and Ray, 2010). While *C. perfringens* is an obligate anaerobe, it can tolerate moderate exposure to air through its capacity to create reducing conditions in its micro-environment (Montville and Mathews, 2008). *C. perfringens* is widely distributed in nature (Lindstrom et al., 2011) and causes two important human illnesses: a relatively mild, classic Type A diarrhea and the more serious Type C human necrotic enteritis (Sigrid and Granum, 2002). *C. perfringens* Type A spores are commonly found in soil and the intestinal tract of humans and food animals (Ryan and Ray, 2010). The *cpe* gene responsible for encoding the *C. perfringens* enterotoxin can be located on the chromosome or the plasmid. Most of the foodborne outbreaks involve Type A isolates carrying the *cpe* gene in the chromosome (Sigrid and Granum, 2002). The chromosomal *cpe* positive strains are resistant to higher concentrations of NaCl, high and low temperatures and nitrites, methods used in meat processing to minimize the risk of foodborne pathogens.

Temperature abuse (slow cooling of cooked products or inadequate reheating) of foods has been identified as a critical factor in a majority of *C. perfringens* related foodborne illness (McClure, 2002). The Food Safety and Inspection Service of the United States Department of Agriculture (USDA-FSIS) compliance guidelines specify that thermally

processed meat and poultry products should be cooled from 54.4°C (130°F) to 26.6°C (80°F) within 1.5 h and from 26.6°C to 4.4°C (40°F) within 5 h. However, cured products can be cooled from 54.4°C to 26.6°C within 5 h and subsequently from 26.6°C to 4.4°C in 10 h (USDA-FSIS, 1999). Cured products such as hams, sausages and hot dogs can support germination and outgrowth of *C. perfringens* spores (Crouch and Golden, 2005). Sodium nitrite is commonly used in meat processing for the development of cured meat color, control oxidation and impart flavor. Approximately 25 % of the sodium nitrite can be detected in cured products as residual nitrite depending on the storage conditions, while the remaining portion is bound to meat components (myoglobin, lipids, proteins) or present in the form of nitric oxide (Honikel, 2008). However, is not clear if the microbial stability and safety of cured meat products is a function of the initial or the residual nitrite content of the food (Roberts, 1996).

The objective of this research was to evaluate the role of sodium nitrite and sodium erythorbate in controlling the germination and outgrowth of *C. perfringens* spores in ham during abusive cooling.

7.3 Materials and methods

C. perfringens cultures: Three different enterotoxin-producing strains of *C. perfringens*, NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and NCTC 10240 (Hobbs serotype 13), were used in the study. The strains were kept as spore stocks in 0.1% sterile peptone water (PW, Difco, Becton Dickinson, Sparks, MD) under refrigeration.

Spore cocktail preparation: The spore crop from each strain of *C. perfringens* was prepared separately, following the protocol outlined by Juneja et al. (1993). Briefly, an

aliquot (0.1 ml) from the stock culture was inoculated into 10 ml of freshly prepared fluid thioglycollate medium (FTM, Difco, Becton Dickinson, Sparks, MD). The *C. perfringens* spores were heat shocked for 20 min at 75°C in a submerged-coil water bath (Isotemp 3013H, Fisher Scientific, Fair Lawn, NJ), cooled in chilled water (4°C) and incubated for 18 h at 37°C. A 1.0 ml portion of this culture was transferred to 10 ml of freshly steamed FTM and incubated for 4 h at 37°C. The fresh culture (1%) was then transferred to modified Duncan-Strong medium and incubated aerobically for 24 h at 37°C. The original Duncan-Strong formulation was modified by replacing starch with 0.4% raffinose (Sigma Chemical Co., St. Louis, MO) and supplemented with 100 mg/ml caffeine (Sigma) to enhance sporulation. The cultures of each strain were then harvested by centrifugation at 7,012 x *g* for 20 min at 4°C (GS-15R, Beckman, Palo Alto, CA) and washed twice with 50 ml of sterile distilled water. The strains were stored as spore stocks in 0.1% sterile peptone water (PW) under refrigeration. The spore cocktail containing all three strains of *C. perfringens* was prepared immediately before the experiments by mixing approximately equal numbers of spores from each spore suspension.

Meat preparation: Fresh pork (gluteus medius) was obtained from the Loeffel Meat Laboratory in the Animal Science Department at the University of Nebraska (Lincoln, NE). The meat was ground once through a 3.175 mm plate of a grinder (Model 4732, Hobart, Troy, OH) and stored at 4°C. Ham ingredients, sodium chloride (2.0% wt/wt), sugar (0.5% wt/wt) and sodium polyphosphate (0.3% wt/wt, Brifisol®512, BK Giulini Corporation, Simmi Valley, CA) calculated as percentage of meat block were dissolved in water (147 mL; 32% of the meat block) to prepare the pickle solution. The pickle solution was added to the ground pork (500 g) while mixing in a bowl mixer (Model K5SSWH, Kitchen Aid, Troy, OH) for 6 min at low speed. Appropriate amounts of sodium nitrite

(Heller Seasonings and Ingredients, Bedford Park, IL) were added to the pickle to provide 0, 50, 100, 150 or 200 ppm in the meat with or without sodium erythorbate (0 or 547 ppm). Ten-gram portions of the meat of each treatment were weighed into vacuum pouches (Nylon; 3-mil standard barrier; water vapor transmission rate of 10 g/liter/m²/24 h at 37.8°C and 100% relative humidity; oxygen transmission rate of 3,000 cm³/liter/m²/24 h at 23°C and 1 atm [101.29 kPa]), measuring 6.25 cm x 10 cm. (Prime Source, Kansas City, MO), vacuum sealed at 12 mbar (1.2 kPa) using a vacuum-packaging machine (A300/H, Multivac, Wolfertschwenden, Germany), and stored at 5°C until use.

Treatments: A 2 (sodium erythorbate concentrations; 0 or 547 ppm) x 5 (sodium nitrite concentrations; 0, 50, 100, 150 or 200 ppm) experimental design was used. Three independent replications as identified by the meat source and the day of preparation were conducted.

Spore inoculation, heat treatment, and cooling procedure: Ten gram portions of ham in the vacuum bag were aseptically inoculated with 100 µl of the three strain *C. perfringens* spore cocktail to attain a spore population of ca. 2.5 log CFU/g of meat. The inoculated samples were vacuum packaged as described, massaged manually for 30 s to evenly distribute the spores, and flattened to a uniform thickness. A pair of bags containing the inoculated meat for each treatment was submerged in a water bath (Isotemp 3013H, Fisher Scientific, Bridgewater, NJ) set at 75°C (167°F) for 20 min to activate the *C. perfringens* spores. After heat treatment, one of the two bags was chilled immediately in an ice water bath, and the *C. perfringens* population was determined. The second bag was transferred into a refrigerated bath with water circulation capabilities

(RTE 740, Thermo Neslab, Portsmouth, NH) set at 54.4°C, allowed to equilibrate to this temperature for 10 min and chilled from 54.4 to 4.0°C exponentially within 15 h.

Enumeration of *C. perfringens* population: The contents of each bag were aseptically transferred to a filter stomacher bag (BagFilter, Spiral Biotech, Norwood, MA). Twenty ml of PW was added, and the contents were stomached for 1 min in a laboratory blender (NEUTEC, Albuquerque, NM). Ten-fold serial dilutions were prepared in PW and appropriate dilutions were either pour plated or spiral plated on tryptose sulfite cycloserine agar (TSC; Oxoid, Ltd., Basingstoke, UK) without egg yolk. After solidification of the agar, plates were overlaid with an additional 8 to 10 ml of TSC and incubated at 35°C for 18 h in an anaerobic chamber (Bactron IV, ShellLab, Cornelius, OR). Typical *C. perfringens* colonies were enumerated and the counts were expressed as log₁₀ CFU/g of meat.

Sample preparation and HPLC analysis: A modified extraction procedure outlined by Hsu et al. (2009) was followed for the determination of nitrite in meat. The modification included the elimination of the heating step before centrifugation. Briefly, each sample (10 g) was blended with 300 ml of distilled water for 1 min and then made up to a final volume of 500 ml in a volumetric flask. Twenty ml of each extract was centrifuged at 5,000 x *g* for 10 min (Model GS-15 R; Beckman instruments, Palo Alto, CA) and the supernatant was filtered through a 0.2 µm filter (Millipore, Bedford MA). The pH of the sample extract was adjusted to 7.0 using 0.1M NaOH (Sigma Aldrich, St. Louis, MO) and stored at 5°C until use. The HPLC equipment used has a tunable absorbance detector (Model 486, Millipore, Milford, MA). An anion chromatographic column (10 µm x 4.1 mm x 250 mm; Hamilton PRP-X100, Phenomenex, Torrance, CA) was used for the separation. Injection volume was of 15 µl with flow rate set at 2.0 ml/min and wavelength

set at 214 nm. The mobile phase was a solution of sodium chloride, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate and acetonitrile (Fisher Scientific, Pittsburg, PA) in MilliQ water. Standard solutions using sodium nitrite (Fisher Scientific, Pittsburg, PA) were prepared in volumetric flasks to provide a range from 0 to 1.50 ppm of nitrite ions to prepare the standard curve. The residual nitrite content in the meat was determined after 3 and 24 h of sample preparation for all the treatments, before heat treatment and after completion of 15 h of exponential cooling.

Water activity and pH measurement: Ten-gram portions of the uncooked sample were homogenized with 25 ml of deionized water for 1 min in the stomacher blender, and the pH of each sample was measured by immersing the electrode of the pH meter (Accumet-Basic/AB15, Fisher Scientific, Bridgewater, NJ) in the sample homogenate. The water activity of the samples was measured using an Aqua Lab 3TE water activity meter (Decagon Devices, Inc., Pullman, WA) following the manufacturer's instructions.

Statistical analysis: Three independent replications were performed for each of the storage times. Data (log CFU/g, residual sodium nitrite, pH and A_w) were compared with analysis of variance of the General Linear Model procedure of the Statistical Analysis System (SAS Institute, Inc., Cary, NC). Fisher's least significant difference (LSD; $\alpha = 0.05$) was used to separate means of the analyzed results.

7.4 Results and Discussion

The pH and water activity of the ham (control) were 6.22 and 0.987, respectively (Table 2.1). The pH of the meat subsequent to *rigor mortis* can be between 5.4 and 5.5.

However, the pH of the muscle can vary depending on the muscle type and the rate of temperature decline subsequent to slaughter (Lawrie, 2006). In the case of the muscle

used for the current study (*gluteus medius*), the normal pH has been reported to be between 5.47 and 6.49 (Jones et al, 2006). The water activity of the pork meat is normally between 0.990 and 0.987 (Singh et al., 2010). Addition of sodium nitrite (50-200 ppm) and/or sodium erythorbate (557 ppm) to the formulation of the ham did not affect ($p>0.05$) the pH or the water activity of the ham.

Exponential cooling of the ham (control) from 54.4°C to 4.4°C (within 3 h of preparation) in 15 h resulted in *C. perfringens* spore germination and outgrowth of 1.46 log CFU/g (Fig. 2.1). Addition of NaNO₂ (50, 100, 150 and 200 ppm) to the ham formulation resulted in *C. perfringens* spore germination and outgrowth of 2.83, 1.61, 2.00 and 0.81 log CFU/g, respectively subsequent to cooling within 15 h. Increasing concentrations of NaNO₂ resulted in greater inhibition ($p<0.05$) of *C. perfringens* spore germination and outgrowth. Inhibition of *C. botulinum* and *C. perfringens* growth by NaNO₂ has been reported in the literature (Christiansen et al., 1973; Moran et al., 1975). Nitrites inhibit *C. botulinum* spore outgrowth rather than germination by the reaction of nitrite with iron containing compounds (e.g., ferredoxin) that are essential for the electron transport, energy production and enzyme activity within the germinated spore (Tompkin, 2005). Riha and Solberg (1975) reported that inhibition of *C. perfringens* by filter-sterilized sodium nitrite may be due to reaction of nitrous acid (from nitrites) with SH-containing constituents of the bacterial cell. In a subsequent report, it was reported that sodium nitrite inhibition of *C. perfringens* may involve an interaction of sodium nitrite as nitrous acid with sulfhydryl-containing constituents of the bacterial cell, resulting in severe restrictions in energy metabolism of the organism (O'Leary and Solberg, 1976).

Addition of sodium erythorbate (557 ppm) to the ham formulation containing NaNO₂ at 50, 100, 150 and 200 ppm resulted in *C. perfringens* populations of 5.22, 3.34, 1.16, and 0.71 log CFU/g, respectively (Fig. 2.1). Addition of sodium erythorbate to the ham formulation containing NaNO₂ resulted in greater increases in *C. perfringens* population compared to the respective treatments containing similar NaNO₂ concentrations alone. These results contrasted with those of previous studies. The role of reducing agents on *C. perfringens* spore germination and outgrowth was contradictory in literature. Erythorbate has been reported to possess antimicrobial activity in meat products (Tompkin et al., 1979; Robinson et al., 1982). Tompkin et al. (1978) demonstrated enhanced antitoxigenic effect of nitrites, even at the lower concentrations, in the presence of sodium isoascorbate (200 ppm) if the product is abused at the time of manufacture. The proposed mechanism for the antitoxigenic activity is the capacity of isoascorbate to sequester iron in meat, making it unavailable for microbial growth (Tompkin, 2005). Tompkin et al. (1979) also reported that isoascorbate can reduce the antitoxigenic efficacy of nitrites after depletion of residual nitrite levels if the product is refrigerated before temperature abuse or excessive amounts of the reducing agent are used. This phenomenon has been demonstrated in freshly prepared meat products (Roberts, 1996). On the other hand, it was also reported that sodium erythorbate did not affect the recovery of *C. perfringens* spores from cured pork formulated with different levels of nitrites (Sauter et al., 1977).

Some meat processing operations formulate and store the meat overnight under refrigeration to enhance uniformity of the ingredients in the meat. Storage of meat over time was shown to reduce the antitoxigenic efficacy of nitrites (Christiansen et al., 1973) due to reduction in the residual nitrite concentration through reaction with meat

components such as myoglobin, secondary amines, amino groups and the thiol groups (Christensen et al., 1973 and Riha and Solberg, 1976). Storage of ham for 24 h prior to heat treatment and subsequent cooling from 54.4°C to 4.4°C resulted in greater increases in *C. perfringens* population compared to the ham processed within 3 h of preparation (Fig. 2.2). Increase in *C. perfringens* spore germination and outgrowth by 4.20 log CFU/g was observed in ham (control) that was stored for 24 h and subsequently cooled within 15 h, compared to 1.46 log CFU/g when the meat was stored for 3 h. Similar increases in *C. perfringens* spore germination and outgrowth were observed subsequent to storage for 24 h in ham containing NaNO₂ and combination of NaNO₂ and sodium erythorbate.

Variations in *C. perfringens* growth under similar cooling conditions and meat formulations have been reported. Singh et al. (2010) reported approximately 5.0 log CFU/g of *C. perfringens* outgrowth in non-cured pork meat formulated with 1% of NaCl and a 20% pump rate after 15 h of cooling. Similarly, Thippareddi et al. (2003) observed *C. perfringens* growth of ca. 3.5 log CFU/g in injected pork formulated with 0.85% of NaCl and a 12% pump rate within 18 h of cooling. On the other hand, <1 log CFU/g growth of *C. perfringens* has been reported in non-cured pork scrapple cooled within 14 h from 54.4°C to 7.2°C, using a final 1% NaCl level and a pH of 6.40 (Juneja et al., 2010). In cured pork products, Zaika (2003) reported *C. perfringens* population increase of 0.4 log CFU/g in ham containing 2.0% of NaCl and 12 ppm of residual sodium nitrite. Amezcuita et al. (2005) reported a *C. perfringens* population increase of ca 1.5 log CFU/g in injected ham formulated with 1.25% of NaCl, 0.3% of phosphates and 156 ppm of sodium nitrite at a 12% pump rate within 15 h of cooling. In our case, the control ham (after 3 h of preparation and packaging) showed lower growth (1.46 log CFU/g)

compared to Amezcuita et al. (2005) even in the absence of nitrites and with a higher pump rate (32%). Variations in *C.perfringens* growth under similar conditions may be explained in terms of the initial inhibition of *C. perfringens* spore germination and outgrowth observed right after ham preparation.

Residual nitrite levels represented 50% of the ingoing NaNO_2 concentration in the formulation for all the treatments. Refrigerated storage of ham for 24 h after sample preparation did not affect ($p>0.05$) the residual nitrite detected in any of the formulations (Fig. 2.3). A combination of factors such as sample pH, storage temperature, presence of reductants and heat treatment can contribute to the variations in residual nitrite concentrations (Honikel, 2008). Dordevic et al. (1980) reported that higher pH values retard the loss of residual nitrite and in some circumstances, a period as long as 60 days was needed to observe a 100% reduction. The author reported immediate loss (50 %) of nitrite in meat with similar pH values compared to the samples in the current study. On the other hand, non-heated products show a lower decline in residual nitrite concentrations compared to heat-treated meat (Gibson et al., 1984). In our study, heat treatment resulted in a reduction ($p\leq 0.05$) in residual nitrite values at higher NaNO_2 concentrations (200 ppm) in the presence of sodium erythorbate (Fig. 2.4). Wootton et al. (1985) reported that freezing of meat results in a significant reduction in the residual nitrite in cured products like ham, salami and corned beef with reductions of 50% after 24 h of frozen (-18°C) storage. Similar decline in residual nitrite concentration was observed when meat was stored at 5°C . Christianssen et al. (1978) reported a reduction of residual nitrite (from 156 to 118 ppm) in cured pork immediately after cooking, with a further decline to 88 ppm after 3 days of storage at 27°C . Hill et al. (1973) demonstrated that

residual nitrite levels were still detectable in frankfurters and sausages after 1 week of storage at 5°C and that this value represented ca. 25% of the initial added nitrite to the meat formulation. Reducing agents such as ascorbates, isoascorbates, cysteine and histidine promote faster reduction of nitrite to nitrous acid and subsequently to nitric oxide (Fox and Nicholas, 1974). Lin et al. (1980) reported significant reduction in residual nitrite concentration in bologna formulated with erythorbate compared to the control samples. In our study, sodium erythorbate reduced ($p \leq 0.05$) the residual nitrite concentration in ham containing the highest NaNO_2 concentration (200 ppm) and was more evident after heat treatment (Fig. 2.3). Conditions used during the experiment including low storage temperature (5°C), short storage time (24 h) and high meat pH (> 6.10) may account for the minimal decline in residual nitrite concentrations. Comparison of residual nitrite reported in literature is complex as it depends on several factors such as chemical reactivity of nitrites, extraction procedures and sensitivity of the methodology (Benjamin and Collins, 1996). HPLC technique, similar to the one used in this study has been reported to be a more sensitive method compared to colorimetric methods for determination of residual nitrite in meat products (Sanderson et al., 1991). Lin et al. (1980) reported lower residual nitrite values from an ingoing NaNO_2 concentration of 156 ppm using a colorimetric method when compared with our study.

Inhibition in *C. perfringens* spore germination and outgrowth in vacuum packaged pork samples after 3 h could not be explained in terms of residual nitrite concentration in the meat. Appreciable reduction in residual nitrite after 24 h was not observed (Fig. 2.3) in the current study. In the absence of other possible inhibitors for *C. perfringens*, the residual oxygen in the meat sample is a possible explanation for the inhibition. The

process of grinding of the meat can incorporate air and oxygen in the product. This oxygen is reduced by the inherent reducing power of meat and the activity of reducing agents such as ascorbates and erythorbate, present during processing of the product. The presence of residual oxygen has been reported previously as a cause of photochemical degradation (discoloration) of vacuum packaged ham during the first 24h of display in illuminated cabinets (Andersen et al., 1990). Residual oxygen in meat products decreases during storage and it is estimated that oxygen concentrations lower than 0.1% are needed to avoid discoloration of cured products (Moller et al., 2000). Residual oxygen concentration of 0.1% has been reported in vacuum packaged meats immediately after packaging (Smiddy et al., 2002), equivalent to an oxygen tension of ca. 0.2 kPa. Additionally, myoglobin in the meat can initiate oxidative reactions leading to accumulation of reactive oxygen species (ROS) including the highly reactive hydroxyl ($\cdot\text{OH}$) radical (Lund et al., 2011). It is possible that the ROS may cause toxicity to *C. perfringens* and inhibit spore germination and outgrowth. However, *C. perfringens* is considered an aerotolerant anaerobe due to the expression of rubrerythrin (*rbr*) and superoxide dismutase (*sod*) genes that confer resistance to oxidative stress (Briolat and Reysset, 2002; Jean et al., 2004). While *C. perfringens* is tolerant to the oxygen (in the air), continuous exposure to ROS in the vacuum packaged meat may inhibit *C. perfringens* spore germination and outgrowth. The inhibitory process is limited in time because the toxicity may dissipate in a vacuum system wherein no additional oxygen is provided, and the meat creates reducing conditions to allow *C. perfringens* spore germination and outgrowth. For this reason, the inhibition of *C. perfringens* spore germination and outgrowth is more evident at the early stages after sample preparation and packaging. The reduced inhibition in samples containing a reducing agent such as

sodium erythorbate supports the involvement of an oxidative mechanism. Reducing agents such as cysteine and ascorbic acid have been reported to prevent *C. perfringens* destruction in cell cultures that are exposed to air (Trinh et al., 2000). Other factors such as the redox potential of the meat could play a role in the observed inhibition of *C. perfringens* spore germination and outgrowth. Early studies demonstrated that redox potential values as high as +350 mV are needed to inhibit *C. perfringens* growth (Pearson and Walker, 1976). Normally, redox potential in vacuum packaged products at day 0 is ca. -91 mV (Kim et al., 2002), a value that would allow *C. perfringens* spore germination and outgrowth. However, oxygen concentration was shown to be a more relevant factor for *C. perfringens* inhibition than redox potential (Walden and Hentges, 1975; Pearson and Walker, 1976).

Inhibition of *C. perfringens* spore germination and outgrowth during cooling of meat products due to the residual oxygen content of the meat has not been reported previously. The initial inhibition of *C. perfringens* growth termed as the “Phoenix effect” has been reported in broth systems (Shoemaker and Pierson, 1976). The “Phoenix effect”, a phenomenon of initial inhibition followed by a significant recovery in the growth of *C. perfringens* in a continuous culture system, is attributed to the application of high temperatures. High temperatures may gradually select for mutants that may survive better at later stages of the growth curve (Mead, 1969). The same phenomenon has been observed during *C. perfringens* spore germination and outgrowth in a variety of meat and poultry products at various temperatures (Amezquita et al., 2005; Huang, 2003; Sanchez-Plata et al., 2005). Shoemaker and Pierson (1976) reported that this phenomenon was not observed when strict anaerobic conditions and pre-reduced media were used. It is possible

that the “Phoenix effect” is partially caused by the presence of oxidative mechanisms resulting in an inhibition of *C. perfringens* growth in the initial stages of growth. Kelly et al. (2003) reported “Phoenix-like” phenomenon similar to the effect observed with *C. perfringens* during the growth of *Campylobacter jejuni* incubated at low temperatures. The authors reported that the inhibition was significantly reduced when the oxygen tension was lowered in the system. Inhibition of *C. perfringens* spore germination and outgrowth in the current study may be explained by oxidative mechanisms similar to those involved in the “Phoenix phenomenon”.

7.5 Conclusions

Refrigerated storage of prepared ham for 24 h before heat treatment (cooking) followed by abusive cooling resulted in greater populations of *C. perfringens* when compared with samples stored during 3 hours. Incorporation of sodium nitrite in the ham formulation resulted in an inhibition of *C. perfringens* spore germination and outgrowth, with higher concentrations exhibiting greater inhibition of *C. perfringens* outgrowth. The concentrations of residual nitrite were not affected by storage at 5°C during 24 h or heat treatment (cooking and cooling). Incorporation of sodium erythorbate in the ham formulation reduced the inhibitory effect of the sodium nitrite in ham that was heat treated and cooled within 3 h of preparation. Residual oxygen in the meat incorporated during grinding and mixing stages could explain the inhibition observed in vacuum packaged ham. The meat processing conditions such as product formulation and preparation procedures (such as length of refrigerated storage) should be replicated while validating the cooling/stabilization processes during manufacture of processed meat products to ensure microbiological safety.

7.6 References

- Amezquita A., Weller C. L., Wang L, Thippareddi H. and Burson D. 2005. Development of an integrated model for heat transfer and dynamic growth of *Clostridium perfringens* during the cooling of cooked boneless ham. *Int. J. Food Microb.* 101: 123-144.
- Andersen H. J., Bertelsen G., Ohlen A. and Skibsted H. 1990. Modified packaging as protection against photodegradation of the colour of pasteurized sliced ham. *Meat Science.* 28: 77-83.
- Benjamin N. and Collins J. 2003. *Food Preservatives* (2 ed). Russell N. and Gould G (Eds.). New York, Kluwer Academic. Page 102.
- Briolat V. and Reysset G. 2002. Identification of the *Clostridium perfringens* genes involved in the adaptative response to oxidative stress. *J. Bacter.* 184: 2333-2343.
- Christiansen L. N., Johnston R. W., Kautter D. A., Howard J. W. and Aunan W. J. 1973. Effect of nitrite and nitrate on toxin production by *Clostridium botulinum* and on nitrosamine formation in perishable canned comminuted cured meat. *Appl. Microb.* 25: 357-362.
- Christiansen L. N., Tompkin R. B. and Shaparis A. B. 1978. Fate of *Clostridium botulinum* in perishable canned cured meat at abuse temperature. *J. Food Prot.* 41: 354-355.
- Crouch E. and Golden N. 2005. A risk assessment for *Clostridium perfringens* in ready-to-eat and partially cooked meat and poultry products. USDA.
- Dordevic V., Vuksan B., Radetic P., Durdica H. and Mitkovic M. 1980. Prilog ispitivanju uticaja pojedinih faktora na promene sadrzaja nitrita u mesu. *Tehnologija mesa.* 21: 287-290.
- Fox J. B. and Nicholas R. A. 1974. Nitrite in Meat. Effect of various compounds on Loss of nitrite. *J. Agr. Food Chem.* 22: 302-306.
- Gibson A. M., Roberts T. A. and Robinson A. 1984. Factors controlling the growth of *Clostridium botulinum* types A and B in pasteurized cured meats VI. Nitrite monitoring during storage of pasteurized pork slurries. *J. Food Techn.* 19: 29-44.
- Gordon G. and Murray A. C. 1988. Effect of pork muscle quality on bacterial growth and retail case life. *Meat Science.* 24: 61-71.
- Hill, L.H., Webb, N.B., Mongol, L.D. and Adams, A.T. 1973. Changes in residual nitrite in sausages and luncheon meat products during storage. *J. Milk Food Technol.* 36: 515-519.
- Honikel K. 2008. The use and control of nitrate and nitrite for the processing of meat products. *Meat Science.* 78: 68-76.
- Huang L. 2003. Dynamic computer simulation of *Clostridium perfringens* growth in cooked ground beef. *Int. J. Food Microb.* 87: 217-227.
- Jean D., Briolat V. and Reysset G. 2004. Oxidative stress response in *Clostridium perfringens*. *Microbiology.* 150: 1649-1659.

- Jones, S.J., Guru, A., Singh, V., and Jones, T.F. 2006. Porcine Myology. Available: <http://porcine.unl.edu>. Accessed on September 30th, 2011.
- Juneja V. K., Call J. E. and Miller A. J. 1993. Evaluation of methylxanthines and related compounds to enhance *Clostridium perfringens* sporulation using Duncan and Strong medium. J. Rapid Methods Automat. Microbiol. 2: 203-218.
- Juneja V. K., Porto Fett A. C., Gartner K., Tufft L. and Luchansky J. B. 2010. Potential for growth of *Clostridium perfringens* from spores in pork scraple during cooling. Foodborne Pathog. Dis. 7: 153-157.
- Kelly A. F., Martinez-Rodriguez A., Bovill R. A. and Mackey B. M. 2003. Description of a “Phoenix” phenomenon in the growth of *Campylobacter jejuni* at temperatures close to the minimum for growth. Appl. Env. Microb. 69: 4975-4978.
- Kim Y. H., Nam K. C. and Ahn D. U. 2002. Color, oxidation-reduction potential and gas production of irradiated meats from different animal species. J. Food Sci. 67: 1692-1695.
- Lawrie R. A. 1998. Lawrie’s Meat Science (6th ed.). Cambridge, Woodhead Publishing. Page 64.
- Lin H., Sebranek J. G., Galloway D. and Lind K. 1980. Effect of sodium erythorbate and packaging conditions of color stability of sliced bologna. J. Food Sc. 45: 115-118.
- Lindstrom M., Heikinheimo A., Lahti P. and Korkeala H. 2011. Novel insights into the epidemiology of *Clostridium perfringens* type A poisoning. Food Microbiology. 28: 192-198.
- Lund M. N., Heinonen M., Baron C. P. and Estevez M. 2011. Protein oxidation in muscle foods: Areview. Mol. Nutr. Food Res. 55: 83-95.
- McClure P. 2002. Foodborne pathogens: hazards, risks analysis and control. Cambridge, Woodhead Publishing. Page 423.
- Mead G. C. 1969. Growth and sporulation of *Clostridium welchii* in breast and leg muscle of poultry. J. Appl. Bacteriol. 32: 86-90.
- Moller J., Jensen J., Olsen M., Skibsted L. and Bertelsen G. 2000. Effect of residual oxygen on colour stability during chill storage of sliced, pasteurized ham packaged in modified atmosphere. Meat Science. 54: 399-405.
- Montville T. and Matthews K. 2008. Food Microbiology: An introduction (2 ed). Washigton D.C., ASM Press. Pages 221-222.
- Moran D. M., Tannembaum S. R. and Archer M. C. 1975. Inhibitor of *Clostridium perfringens* formed by heating sodium nitrite in a chemically defined medium. Appl. Microb. 30: 838-843.
- O’Leary V. and Solberg M. 1976. Effect of sodium nitrite inhibition on intracellular thiol groups and on the activity of certain glycolytic enzymes in *Clostridium perfringens*. Appl. and Env. Microb. 31: 208-212.

- Pearson C. B. and Walker H. W. 1976. Effect of Oxidation-Reduction potential upon growth and sporulation of *Clostridium perfringens*. J. Milk Food Technol. 39: 421-425.
- Riha W. E. and Solberg M. 1975. *Clostridium perfringens* growth in a nitrite containing defined medium sterilized by heat or filtration. J. Food Sci. 40: 443.
- Roberts T. A. and Dainty R. H. 1996. Nitrates and nitrites in Food and Water. Hill M. J (Ed.). Cambridge, Woodhead Publishing. Pages 117-130.
- Robinson A., Gibson A. M. and Roberts T. A. 1982 Factors controlling the growth of *Clostridium botulinum* types A and B in pasteurized meats. V. Prediction of toxin production: non-linear effects of storage temperature and salt concentration. J. Food Techn. 17: 727-744.
- Ryan K. and Ray C. 2010. Sherris Medical Microbiology (5ed). New York, McGraw Hill. Pages 520-521.
- Sanchez-Plata M. X., Amezcua A., Blankenship E., Burson D., Juneja V. K. and Thippareddi H. 2005. Predictive model for *Clostridium perfringens* growth in roast beef during cooling and inhibition of spore germination and outgrowth by organic acid salts. J. Food Prot. 68: 2594-2605.
- Sanderson J. E., Ross J. and Lee K. 1991. Nitrite analysis in meats: comparison of two methods. J. Food Sci. 56: 1123-1124.
- Sauter E. A., James D. K. and Langlois B. E. 1977. Effect of nitrite and erythorbate on recovery of *Clostridium perfringens* spores in cured pork. J. Food Sci. 42: 1678-1679.
- Shoemaker S. P. and Pierson M. D. 1976. "Phoenix phenomenon" in the growth of *Clostridium perfringens*. Appl. Env. Microb. 32: 803-807.
- Sigrid B. and Granum P. 2002. *Clostridium perfringens* and foodborne infections. Int. J. Food Mic. 74: 195-202.
- Singh A., Korasapati N. R., Juneja V. K. and Thippareddi H. 2010. Effect of phosphate meat (pork) types on the germination and outgrowth of *Clostridium perfringens* spores during abusive chilling. J. Food Prot. 73: 879-887.
- Smiddy M., Fitzgerald M., Kerry J. P., Papkovsky D. B., O'Sullivan C. K. and Guilbault G. G. 2002. Use of oxygen sensors to non-destructively measure the oxygen content in modified atmosphere and vacuum-packed beef: impact of oxygen content on lipid oxidation. Meat Science. 61: 285-290.
- Thippareddi H., Juneja V.K., Phebus R., Marsden J.L. and Kastner C.L. 2003. Control of *Clostridium perfringens* germination and outgrowth by buffered sodium citrate during chilling of roast beef and injected pork. J. Food Prot. 66:376-381.
- Tompkin R. B. 2005. Antimicrobials in Food. Davidson P. M., Sofos J. N. and Branen A (Eds.). L. Florida, Taylor & Francis Group. Page 170-171.
- Tompkin R. B., Christiansen L. N. and Shaparis A. B. 1978. Enhancing nitrite inhibition of *Clostridium botulinum* with isoascorbate in perishable canned cured meat. Appl. Env. Microb. 35: 59-61

- Tompkin R. B., Christiansen L. N. and Shaparis A. B. 1979. Iron and the antitoxin efficacy of nitrite. *Appl. and Env. Microb.* 37: 351-353.
- Trinh S., Briolat V., and Reysset G. 2000. Growth response of *Clostridium perfringens* to oxidative stress. *Anaerobe.* 6: 233-240.
- USDA-FSIS. 1999. Performance standards for the production of certain meat and poultry products. FSIS Directive 7111.1 Federal Register 64:732-749.
- Walden W. C. and Hentgens D. J. 1975. Differential effects of oxygen and oxidation-reduction potential on the multiplication of three species of anaerobic intestinal bacteria. *Applied Microb.* 30: 781-785.
- Wootton M., Kok S. H. and Buckle K. A. 1985. Determination of nitrite and nitrate levels in meat and vegetable products by high performance liquid chromatography. *J. Sci. Food Agric.* 36: 297-304.
- Zaika L. L. 2003. Influence of NaCl content and cooling rate on outgrowth of *Clostridium perfringens* spores in cooked ham and beef. *J. Food Prot.* 66: 1599-1603.

7.7 List of Tables

Table 2.1. Mean pH (\pm SD) and water activity values of ham containing different concentrations of sodium nitrite (from 0 to 200 ppm) and sodium erythorbate (0 and 557 ppm).

Treatment		pH ^a	Water activity
NaNO ₂	Sodium erythorbate		
-	-	6.22 \pm 0.09 ^b	0.987 \pm 0.000 ^{b,c}
50	-	6.24 \pm 0.14 ^b	0.985 \pm 0.003 ^{b,c}
100	-	6.23 \pm 0.11 ^b	0.983 \pm 0.005 ^{b,c}
150	-	6.23 \pm 0.08 ^b	0.984 \pm 0.008 ^c
200	-	6.26 \pm 0.03 ^b	0.985 \pm 0.002 ^c
50	557	6.23 \pm 0.15 ^b	0.985 \pm 0.004 ^b
100	557	6.26 \pm 0.14 ^b	0.985 \pm 0.003 ^{b,c}
150	557	6.30 \pm 0.15 ^b	0.983 \pm 0.003 ^{b,c}
200	557	6.25 \pm 0.18 ^b	0.986 \pm 0.004 ^{b,c}

^a Mean \pm standard deviations; values represent three independent replications and triplicate measurements for each sample.

^b Values followed by the same letter are not statistically different.

7.8 Legend to the Figures

- Fig. 2.1. Mean *Clostridium perfringens* populations (log CFU/g) in ham containing various concentrations of NaNO₂ and sodium erythorbate prepared and processed within 3 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 15 h (Chill).
- Fig. 2.2. Mean *Clostridium perfringens* populations (log CFU/g) in ham containing various concentrations of NaNO₂ and sodium erythorbate prepared and processed within 24 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 15 h (Chill).
- Fig. 2.3. Mean residual nitrite levels (ppm) expressed as the total curing ingredient in ham (prepared and processed within 3 and 24 h) before heat treatment (cooking at 75°C for 20 min followed by cooling exponentially from 54.4°C to 4°C in 15 h).
- Fig. 2.4. Mean residual nitrite levels (ppm) expressed as the total curing ingredient in ham (prepared and processed 24 h) before and after heat treatment (cooking at 75°C for 20 min followed by cooling exponentially from 54.4°C to 4°C in 15 h).

Fig. 2.1

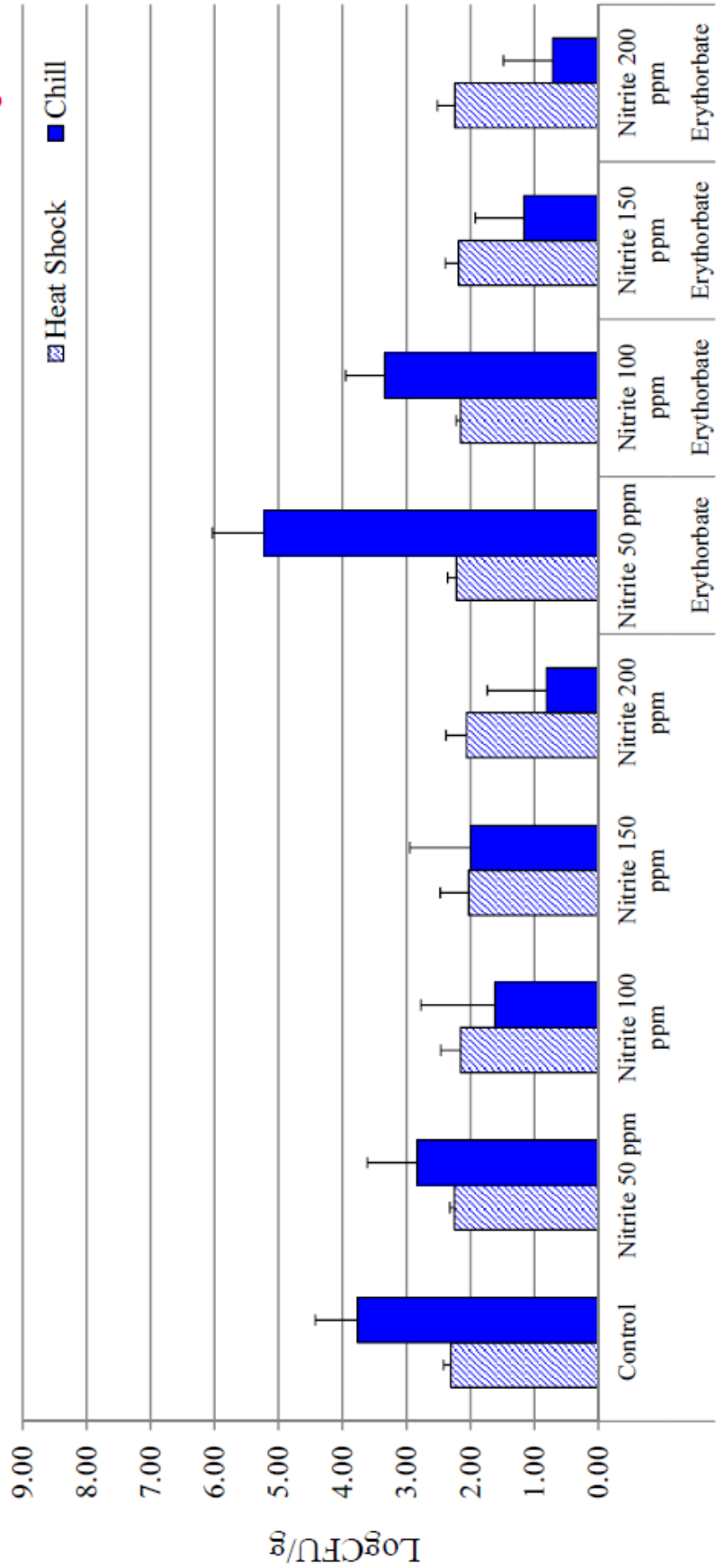


Fig. 2.2

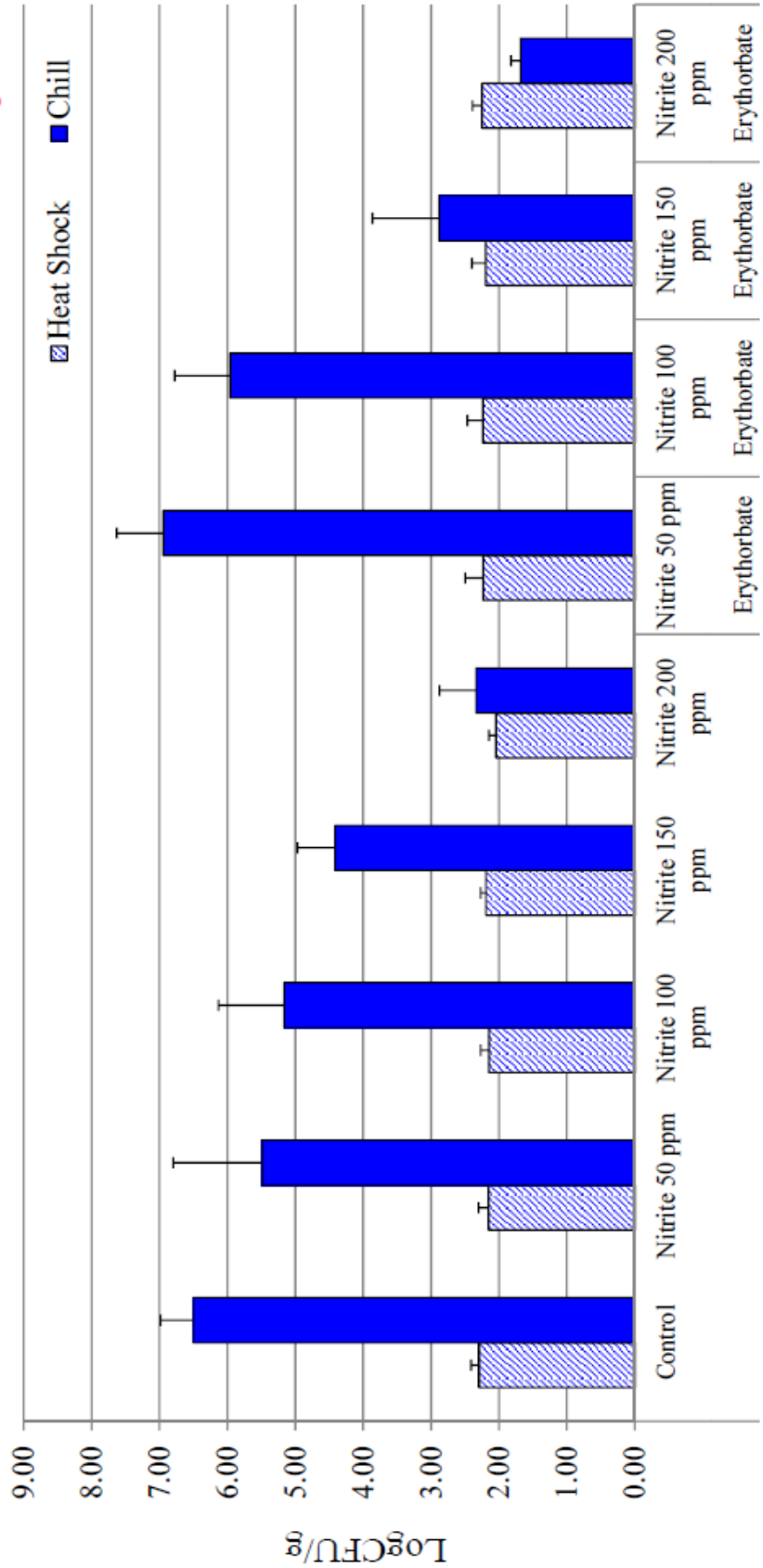


Fig. 2.3

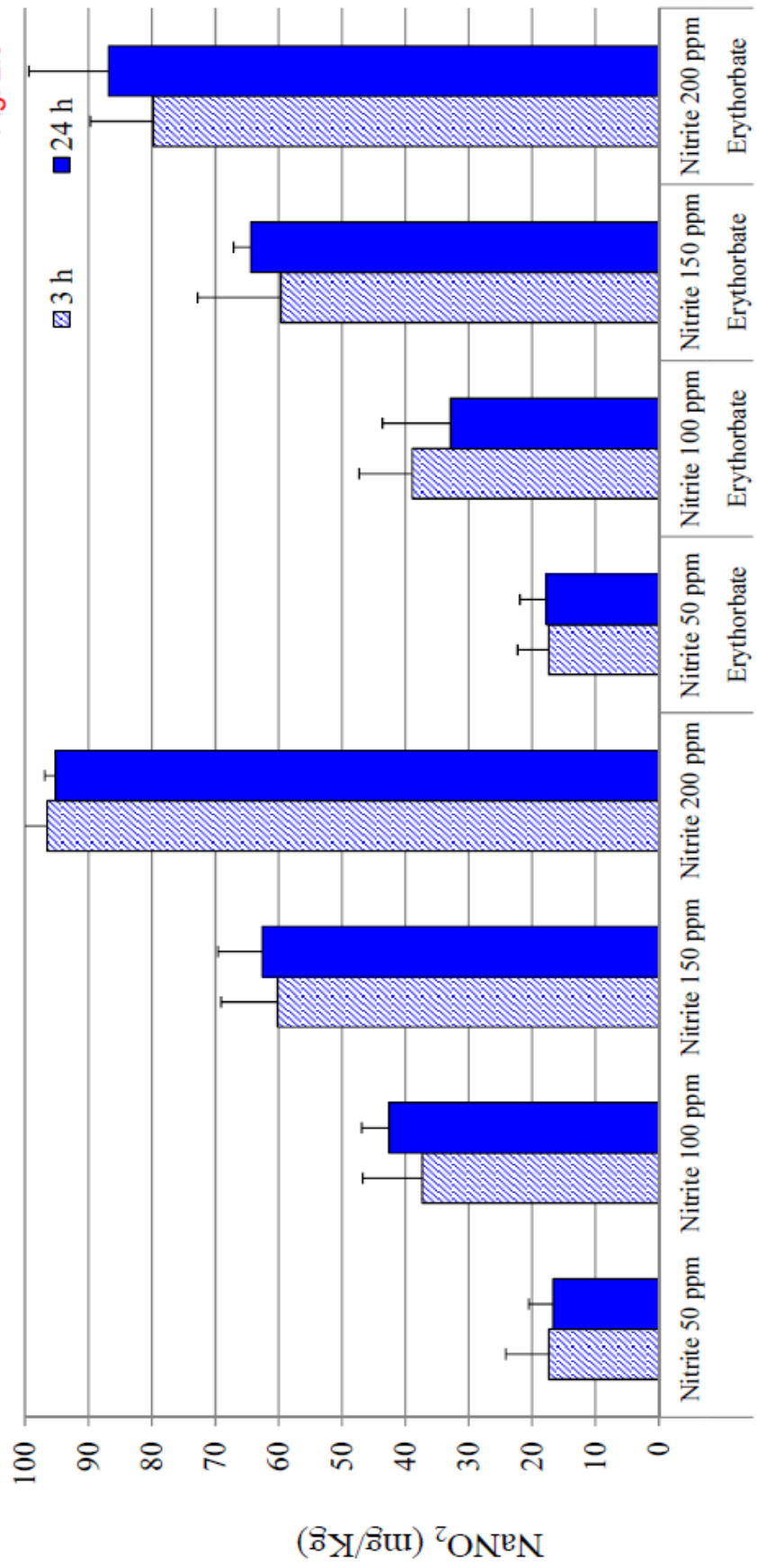
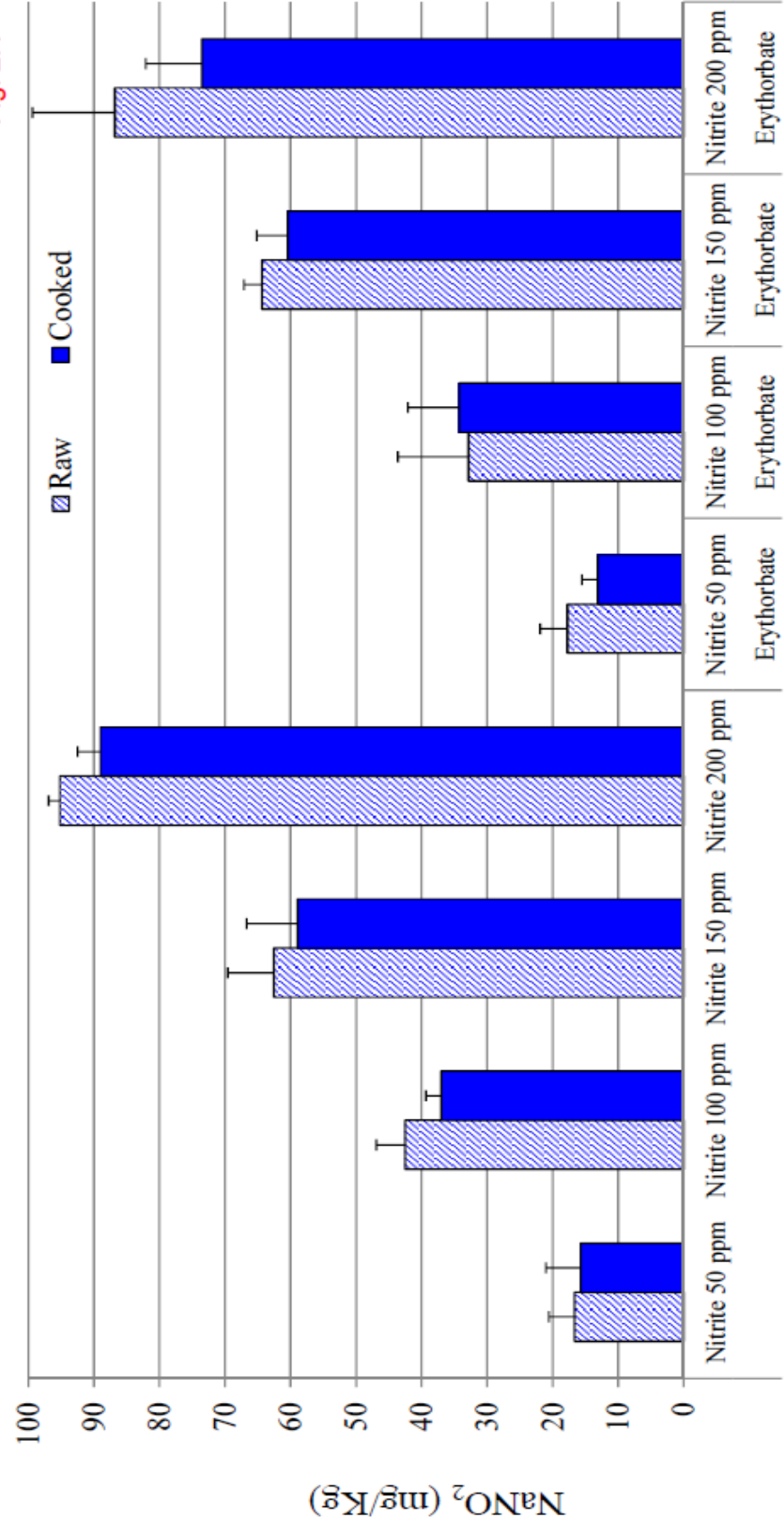


Fig. 2.4



CHAPTER 3

8. Control of *Clostridium perfringens* Spore Germination and Outgrowth by Potassium Lactate and Sodium Diacetate in Ham Containing Reduced Sodium Chloride

8.1 ABSTRACT

Control of *C. perfringens* spore germination and outgrowth by a mixture of potassium lactate and sodium diacetate (*Opti.Form* PD4®) in reduced sodium ham under abusive cooling was evaluated. Ground pork was mixed with NaNO₂ (0 or 100 ppm), NaCl (1 or 2%) and *Opti.Form* PD4® (0, 1.5 or 2.5 %). A portion of the meat (10 g) was transferred to a vacuum bag and inoculated with a three-strain *C. perfringens* spore cocktail to obtain a spore population of ca. 2.5 log spores/g. Samples were vacuum packed, heat treated (75°C, 20 min) and cooled from 54.4°C to 4.4°C within 9, 15 or 21 h after 3 or 24 h of ham preparation. In samples processed after 3 h, *C. perfringens* population increases of 5.18 and 4.26 log CFU/g were observed in non-cured ham (control) formulated with 1 and 2 % of NaCl, respectively and cooled exponentially within 21 h. Incorporation of *Opti.Form* PD4® (1.5%) inhibited *C. perfringens* spore germination and outgrowth regardless of the cooling rate (9, 15 or 21 h). Addition of NaNO₂ enhanced the antimicrobial activity of organic acid salts. Heat treatment and cooling of ham after 24 h of preparation resulted in greater populations of *C. perfringens*. The incorporation of NaNO₂, organic acid salts and shorter time periods between sample preparation and heat treatment/cooling resulted in greater inhibition of *C. perfringens* spore germination and outgrowth.

Key words: sodium chloride, sodium nitrite, *Opti.Form* PD4®, *Clostridium perfringens*

8.2 Introduction

Clostridium perfringens is a Gram positive, large, non-motile bacterium with the ability to form spores that are highly resistant to environmental conditions (Ryan and Ray, 2010).

C. perfringens is widely distributed in nature (Lindstrom et al., 2011) and can cause two important foodborne diseases, the relatively mild classic Type A diarrhea and the more serious Type C human necrotic enteritis (Sigrid and Granum, 2002). The strains involved in Type A outbreaks are more resistant to high concentrations of NaCl and NaNO₂, extreme temperatures and other extrinsic factors used in meat processing to minimize the risk of foodborne pathogens.

Temperature abuse (slow cooling of cooked products or inadequate reheating of cooled products) provides conditions that are conducive for *C. perfringens* spore germination and outgrowth and subsequent multiplication to hazardous levels (McClure, 2002). In broth media, a minimum concentration of 2.5% of NaCl and 0.4% (4,000 ppm) of sodium nitrite is necessary to inhibit the growth of *C. perfringens* vegetative cells carrying the enterotoxin gene (*cpe*) in the chromosome to < 1 log CFU/ml (Li and McClane 2006). However, in cured products and salted seafood, *C. perfringens* can survive and grow at higher (3%) NaCl concentrations (Baros, 2001). Meat processors use several ingredients with antimicrobial activity to control pathogen growth in meat products. The Food Safety and Inspection Service of the United States Department of Agriculture (USDA-FSIS) has approved the use of some antimicrobial ingredients like citrates and lactates for meat and poultry products (USDA-FSIS, 2000). Ingredients like sodium and potassium salts of organic acids, including citric, acetic and propionic acid are effective controlling the germination and outgrowth of *C. perfringens* spores (USDA-FSIS, 2000). During

abusive cooling of meat and poultry products, sodium chloride and sodium nitrite alone may not be adequate to control *C. perfringens* spore germination and outgrowth. Use of organic acid salts in meat formulations can be a practical alternative for meat processors to minimize *C. perfringens* spore germination and outgrowth.

Sodium chloride is the major contributor of sodium intake in the human diet (Desmond, 2006). High blood pressure (hypertension) has been identified as one of the consequences of excessive sodium intake in developed countries. Hypertension can lead to cardiovascular disease (CVD) in 80% of all the cases and is involved in 62% of all the cases of strokes and 42% of coronary heart diseases (CHD) (He and McGregor, 2008). CVD is the leading cause of death worldwide and the second leading cause of disability just behind the malnutrition. The World Health Organization (WHO) estimates that 80% of all adults are at risk of CVD from hypertension and was projected to cause more than 26 million deaths by the year 2030 (McKay and Mensah, 2004). The American Medical Association (AMA) has estimated premature deaths of 150,000 in the United States, every year due to hypertension. Currently, WHO is recommending reformulation of processed and prepared foods to achieve lower levels of sodium in the human diet. Salt (NaCl) is a common ingredient in most processed meat and poultry products and contributes several functions in food systems including antimicrobial activity, and hence its reduction is considered a challenge for the meat industry (Desmond, 2006). Reduction in NaCl levels should be based on microbiological validation of the safety of the product containing reduced sodium concentrations (Stringer and Pin, 2005). A strategy to reduce sodium in meat products is to substitute NaCl with potassium salts or non-chloride salts such as organic acid salts at low concentrations.

The objective of the study was to evaluate the effect of organic acid salts (potassium lactate and sodium diacetate), sodium nitrite and sodium chloride on the germination and outgrowth of *C. perfringens* spores in ham during abusive cooling.

8.3 Materials and Methods

C. perfringens cultures: Three enterotoxin-producing strains of *C. perfringens*, NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and NCTC 10240 (Hobbs serotype 13) were used in the study. The strains were stored as spore stocks in 0.1% sterile peptone water (PW, Difco, Becton Dickinson, Sparks, MD) under refrigeration conditions.

Spore cocktail preparation: The spore crop from *C. perfringens* strain was prepared separately, following the protocol outlined by Juneja et al. (1993). Briefly, an aliquot (0.1 ml) from the stock culture was inoculated into 10 ml of freshly prepared fluid thioglycollate medium (FTG, Difco, Becton Dickinson, Sparks, MD). The *C. perfringens* spores in FTG were heat activated for 20 min at 75°C in a submerged-coil water bath (Isotemp 3013H, Fisher Scientific, Fair Lawn, NJ), cooled in chilled water, and incubated for 18 h at 37°C. A 1.0 ml portion of this culture was transferred to 10 ml of freshly steamed FTG medium and incubated for 4 h at 37°C. The fresh culture (1%) was then transferred to modified Duncan-Strong medium and incubated aerobically for 24 h at 37°C. The original Duncan-Strong formulation was modified by replacing starch with 0.4% raffinose (Sigma Chemical Co., St. Louis, MO) and supplemented with 100 mg/ml of caffeine (Sigma) to enhance sporulation. The spores of each strain were then harvested by centrifugation at 7,012 x *g* for 20 min at 4°C (GS-15R, Beckman, Palo Alto, CA) and washed twice with 50 ml of sterile distilled water. The spore stocks were stored in 0.1%

sterile peptone water (PW) under refrigeration. A spore cocktail containing all three strains of *C. perfringens* was prepared immediately before the experiments by mixing approximately equal numbers of spores from each spore suspension.

Meat preparation: Fresh pork (IMPS 402G) (USDA, 2010) was obtained from the Loeffel Meat Laboratory in the Animal Science Department at the University of Nebraska (Lincoln, NE). The meat was ground once through a 3.175 mm plate of a grinder (Model 4732, Hobart, Troy, OH). Ham ingredients, sugar (0.5% wt/wt), sodium polyphosphate (0.3% wt/wt, Brifisol®512, BK Giulini Corporation, Simmi Valley, CA) and sodium erythorbate (547 ppm for cured samples) (Herstelle, Werl Ladenburg, Germany) calculated as percentage of meat block were dissolved in water (147 mL; 32% of the meat block) for the control product. The pickle solution was added to the meat (500 g) while mixing in a bowl mixer (Model K5SSWH, Kitchen Aid, Troy, OH) for 6 min at low speed. Appropriate amounts of the pickle were added to provide two concentrations of sodium nitrite (0 or 100 ppm), two concentrations of NaCl (1 or 2%) and three concentrations (0, 1.5 or 2.5%) of a mixture of potassium lactate and sodium diacetate (*Opti.Form* PD4®, PURAC, Lincolnshire, IL). Ten-gram portions of each treatment were weighed into vacuum bags (Nylon; 3-mil standard barrier; water vapor transmission rate of 10 g/liter/m²/24 h at 37.8°C and 100% relative humidity; oxygen transmission rate of 3,000 cm³/liter/m²/24 h at 23°C and 1 atm [101.29 kPa]), measuring 6.25 cm x 10 cm (Prime Source, Kansas City, MO), vacuum sealed at 12 mbar (1.2 kPa) using a vacuum-packaging machine (A300/H, Multivac, Wolfertschwenden, Germany) and stored at 5°C until use.

Treatments: A 2 (sodium chloride concentrations; 1 or 2 %) x 2 (sodium nitrite concentrations; 0 or 100 ppm) x 3 (*Opti.Form* PD4® concentrations; 0, 1.5 or 2.5%) experimental design was used. Three independent replications as identified by meat source and the day of preparation were conducted.

Spore inoculation, heat treatment, and cooling procedure: Ten gram portions of ham were aseptically inoculated with 100 µl of the three strain spore cocktail of *C. perfringens* to attain a spore population of ca. 2.5 log spores/g of meat. The inoculated samples were vacuum packaged as described, massaged manually for 30 s to evenly distribute the spores, and flattened to a uniform thickness. A pair of bags containing the inoculated meat for each treatment was submerged into a water bath (Isotemp 3013H, Fisher Scientific, Bridgewater, NJ) for 20 min at 75°C to activate the *C. perfringens* spores. After heat treatment, one of the two bags was cooled immediately in an ice water bath and the *C. perfringens* population was determined. The second bag was transferred to a refrigerated bath with water circulation capabilities (RTE 740, Thermo Neslab, Portsmouth, NH) set at 54.4°C; allowed to equilibrate to this temperature for 10 min; and cooled from 54.4°C to 4.4°C exponentially within 9, 15 or 21 h. The heat treatment and cooling procedure was performed within 3 and 24 h of meat sample preparation.

Enumeration of *C. perfringens* population: The contents of each bag were aseptically transferred to a filter stomacher bag (BagFilter, Spiral Biotech, Norwood, MA). Twenty ml PW was added, and the contents were stomached for 1 min in a laboratory blender (NEUTEC, Albuquerque, NM). Ten-fold serial dilutions were prepared in 0.1% PW and appropriate dilutions were either pour plated or spiral plated on tryptose-sulfite-cycloserine agar (TSC; Oxoid, Ltd., Basingstoke, UK) without egg yolk. After solidification of the agar, plates were overlaid with an additional 8 to 10 ml of TSC and

incubated at 35°C for 18 h in an anaerobic chamber (Bactron IV, Shel Lab, Cornelius, OR). Typical *C. perfringens* colonies were enumerated, and the counts were expressed as log₁₀ CFU/g of meat.

Water activity and pH measurement: Ten-gram portions of the non-heat treated sample were homogenized with 25 ml of deionized water for 1 min in the stomacher blender, and the pH of each sample was measured by immersing the electrode of the pH meter (model Accumet-Basic/AB15, Fisher Scientific) in the sample homogenate. The water activity of the samples was measured with an Aqua Lab 3TE water activity meter (Decagon Devices, Inc., Pullman, WA), following the manufacturer's instructions.

Statistical analysis: Three independent replications were performed for each of the three exponential cooling times. Data (log CFU/g, pH and A_w) were analyzed, and the results of each treatment were compared using analysis of variance of the General Linear Model procedure of the Statistical Analysis System (SAS Institute, Inc., Cary, NC). Fisher's least significant difference (LSD; $\alpha = 0.05$) was used to separate the analyzed results.

8.4 Results and Discussion

The pH of the ham (all treatments) was between 5.69 and 5.82 (Table 3.1). These results are consistent with pH values reported for pork meat. Singh et al. (2010) reported pH values between 5.69 and 5.92 for pork samples formulated with different concentrations of phosphates. Incorporation of sodium nitrite and a mixture of organic acid salts (potassium lactate and sodium diacetate) did not affect ($p > 0.05$) the pH values of the ham. Thippareddi et al. (2003) reported that the addition of sodium diacetate did not affect the pH of roast beef or injected pork. Devlieghere et al. (2009) reported minimal changes in product pH when different amounts of *Opti.Form* PD4® were included in the

formulation of cooked ham. The water activity values (A_w) of the ham were between 0.994 and 0.984 (Table 3.1). Increasing *Opti.Form* PD4® concentration to 2.5% resulted in a reduction ($p \leq 0.05$) in water activity of ham when compared to the control (no added *Opti.Form* PD4®). Lactates are hygroscopic and can bind water in meat and poultry products (Abou-Zeid et al., 2007). The addition of a mixture of sodium lactate and sodium diacetate can decrease the non-bound free water of cooked, boneless ham with a significant impact on quality and sensory attributes (Poovey et al., 2008). Reduction of water activity has been reported as a mechanism for inhibition of microbial growth by lactate salts in food products (Abou-Zeid et al., 2007).

Minimal *C. perfringens* spore germination and outgrowth was observed in ham samples cooled within 9.0 h from 54.4°C to 4.4°C after 3 h of sample preparation and packaging (Fig. 3.1). Total increases in *C. perfringens* populations of 0.26 and 0.33 log CFU/g were observed for control non-cured ham containing 1 and 2% of sodium chloride, respectively. The addition of either a mixture of organic acid salts or sodium nitrite resulted in lower populations of *C. perfringens* in ham subsequent to cooling. A final *C. perfringens* outgrowth of < 1.0 log CFU/g after cooling is consistent with the USDA-FSIS stabilization performance standards for meat and poultry products (USDA-FSIS, 1999). Extension of cooling time to 15 h resulted in *C. perfringens* spore germination and outgrowth of 2.39 and 1.99 log CFU/g in ham (no nitrite) containing NaCl concentrations of 1 and 2%, respectively (Fig. 3.2). Similarly, incorporation of NaNO₂ or *Opti.Form* PD4® resulted in lower *C. perfringens* populations compared to the initial level (2.5 log CFU/g). NaCl can control microbial growth in foods through the reduction of water activity and alteration of enzyme metabolism (Taormina, 2010). Increasing sodium

chloride concentration in ham inhibited *C. perfringens* spore germination and outgrowth under abusive cooling conditions (Zaika, 2003). *C. perfringens* outgrowth of 1.04 log CFU/g was observed for ham containing 1% of NaCl and 1.5% of *Opti.Form* PD4® after 21 h of cooling (Fig. 3.3). No growth of *C. perfringens* populations was reported in other treatments of ham meat containing *Opti.Form* PD4® or NaNO₂.

Potassium lactate is a generally recognized as safe (GRAS) substance that is used in foods to improve texture, regulate acidity and prolong shelf life (Theron and Rykers, 2011).

Sodium and potassium lactates can be used at levels up to 4 % in meat products (USDA-FSIS, 2000). Antimicrobial activity of lactates can be explained by the reduction in water activity of the product, the effect of the lactate ion from the salt on the bacterial metabolism (Abou-Zeid et al., 2007) and the inhibition of proline dehydrogenase activity (Apostolidis et al., 2008). Similarly, sodium diacetate is used as a food additive to improve flavor and microbial stability of meat products. Although the antimicrobial mechanism of diacetate (sodium acetate plus acetic acid) is not clear, evidence indicates that inhibitory activity is related to the dissociation constant of acetic acid (pKa value of 4.76). Due to its higher pKa, the concentration of the undissociated form of the acid is high at the normal pH values in meat samples (*ca.* 5.4-5.5) in comparison with other organic acids (Vasseur et al., 1999). The undissociated acetic acid can pass through bacterial membranes to acidify the cytoplasm and interfere with metabolic activity. Sodium diacetate in combination with lactates is considered as an important antimicrobial hurdle for inhibition of *Listeria monocytogenes* growth in processed meat and poultry products (Abou-Zeid et al., 2007; Juneja, 2003).

Organic acids salts have been reported to be effective for inhibition of germination and outgrowth of anaerobic spore formers such as *C. perfringens* and *C. botulinum* in meat and poultry products. Sodium diacetate in combination with buffered sodium citrate (1%) was effective in controlling *C. perfringens* spore germination and outgrowth after 21 h of cooling in roast beef and injected pork (12% pump rate, 0.85% of NaCl) (Thippareddi et al., 2003). Juneja and Thippareddi (2004a) reported inhibition of *C. perfringens* spore germination and outgrowth after 18 h of exponential cooling using a minimum concentrations of 2% of sodium lactate or sodium acetate. Sabah et al. (2003) reported that sodium lactate (2%) and sodium diacetate (0.25%) could prevent *C. perfringens* outgrowth in vacuum-packaged roast beef (1.5% NaCl) cooled within 18 h. In the current study, *Opti.Form* PD4® concentration of 1.5% was not effective in preventing *C. perfringens* outgrowth after 21 h of cooling in non-cured ham containing a lower NaCl concentration (Fig. 3.3). Juneja and Thippareddi (2004b) reported that a mixture of sodium lactate and sodium diacetate (1.5%) was effective in controlling *C. perfringens* spore germination and outgrowth after 21 h of cooling in roast beef (0.85% of NaCl). Lower inhibition of *C. perfringens* growth observed in the current study at similar concentration of sodium lactate and sodium diacetate (1.5%) may be due to the use of a higher pump rate (32%), resulting in greater moisture content of the product.

Addition of minimal amounts of sodium nitrite (100 ppm) inhibited *C. perfringens* spore germination and outgrowth subsequent to temperature abuse in the presence of organic acid salts. The antimicrobial activity of nitrites against *C. perfringens* is related with the reaction of nitrous acid with molecules containing SH-groups such as glyceraldehyde-3-phosphate-dehydrogenase and aldolase (O'Leary and Solberg, 1976). Also, nitrites can

interfere with iron containing molecules such as ferredoxin required for electron transport, energy production and enzyme activity within the germinated spore (Tompkin, 2005). Nitrites (156 ppm), in combination with high levels of *Opti.Form* PD4® and 2% of NaCl inhibited the germination and outgrowth of *C. perfringens* spores in ham and frankfurters during storage at 22°C for 10 days (Jackson et al., 2011). Reduction of nitrite concentration (from 100 ppm to 0 ppm) reduced the antimicrobial efficacy of organic acid salts during abusive cooling conditions. Nevertheless, mixtures of sodium diacetate and lactic acid salts (calcium, potassium and sodium) can control *C. perfringens* outgrowth in pork after 21 h of abusive cooling even in the total absence of sodium nitrite (Thippareddi et al., 2003; Velugoti et al., 2007). The additive effect of nitrites and organic acid salts in controlling *C. perfringens* spore germination and outgrowth allows for a further reduction in the salt content of cured products. Sodium nitrite in meat products contributes to the development of the cured color, improves taste and controls microbial growth (Honikel, 2008). However, consumers are increasingly interested in “preservative-free” and “healthier” foods where concentrations and types of non-meat ingredients such as salt and sodium nitrite are reduced (Jackson et al., 2011). Incorporation of *Opti.Form* PD4® (1.5 %) allowed reduction in the salt and sodium nitrite in ham with minimal impact on *C. perfringens* germination and outgrowth when replicating industry practice of short storage period after preparation and packaging (3 h prior to heat treatment and cooling).

In most of the previous research on germination and outgrowth of *C. perfringens* spores, protocols involved preparation and storage of the meat products under freezing conditions (-20°C) for an unspecified period of time before use (Amezquita et al., 2005; Singh et al., 2010; Huang, 2003; Zaika, 2003; Juneja et al., 2006). In cured meat products, storage of

the meat (refrigerated or frozen) can result in a reduction of residual nitrite content and consequently, loss of antimicrobial activity (Hill et al., 1973; Christiansen et al., 1973). Also, residual oxygen in vacuum packaged samples is also reduced over time during storage (Moller et al., 2000), resulting in greater *C. perfringens* spore germination and outgrowth during abusive cooling. The specific changes in meat characteristics during storage under refrigeration or freezing conditions that affect *C. perfringens* spore germination and outgrowth after abusive cooling conditions have not been elucidated in literature.

Greater populations of *C. perfringens* were observed in all treatments ($p > 0.05$) that were heat treated and cooled after 24 h of preparation compared to those that were processed within 3 h of preparation during abusive cooling within 15 h (Fig. 3.4). Germination and outgrowth of *C. perfringens* spores ($> 4 \log \text{CFU/g}$) was observed in samples containing either 1 or 2% NaCl (no nitrite or *Opti.Form* PD4®), whereas minimal growth ($< 1.0 \log \text{CFU/g}$) was observed in all other treatments, in product processed within 24 h of preparation.

Greater *C. perfringens* populations were observed in ham exponentially cooled within 21 h compared to 15 h, for the respective treatments (Fig. 3.5). Increasing concentration of NaCl (from 1 to 2%), incorporation of sodium nitrite (100 ppm) and increasing concentrations of *Opti.form* PD4® (0, 1.5 and 2.5%) resulted in inhibition of *C. perfringens* populations during exponential cooling of ham prepared and processed within 24 h. However, the inhibition of *C. perfringens* spore germination and outgrowth was lower in ham prepared and processed within 24 h compared to ham prepared and processed within 3 h.

The product preparation and processing (time between preparation and processing) affect *C. perfringens* spore germination and outgrowth, for the same product formulations.

Thus, care should be exercised in making comparisons of *C. perfringens* spore germination and outgrowth between different studies reported in literature.

Other transient inhibitory events have been previously reported during *C. perfringens* growth (Shoemaker and Pierson, 1976). An initial decline in *C. perfringens* populations and extended lag phase duration (phoenix effect) were observed when the laboratory media were inoculated with the organism and held at temperatures $>51^{\circ}\text{C}$. The authors attributed this phenomenon to the higher temperature of incubation as well as the presence of oxygen in the laboratory medium. Subsequent growth of *C. perfringens* results in creation of reducing conditions in the medium, conducive for faster growth of the organism. Vacuum packaging and storage of prepared ham for 24 h (compared to 3 h of storage) probably resulted in creation of reducing conditions in the meat, conducive for greater growth of *C. perfringens*.

Variations in sample preparation and holding under refrigeration or frozen conditions may explain the differences in *C. perfringens* spore germination and outgrowth in product with similar meat formulations reported in the literature. Juneja and Thippareddi (2004b) reported that 2% sodium lactate was effective in controlling *C. perfringens* spore germination and outgrowth during abusive cooling within 21 h in turkey breast (0.85% NaCl). However, Velugoti et al. (2007) reported that sodium lactate (2%) was not effective in controlling *C. perfringens* spore germination and outgrowth in injected turkey (0.85% of NaCl) after 21 h of cooling. Care should be exercised in comparing *C. perfringens* spore germination and outgrowth in meat and meat products as the procedures

followed during preparation and storage history of the product can have a significant impact on the growth of the organism.

8.5 Conclusions

Reduction of NaCl content in ham increased the risk for *C. perfringens* spore germination and outgrowth during abusive cooling conditions. The addition of antimicrobials such as sodium nitrite and salts of organic acids in the ham formulation can achieve further sodium reductions. Use of potassium lactate and sodium diacetate (*Opti.Form* PD4® at 1.5%) was effective in controlling *C. perfringens* spore germination and outgrowth under abusive cooling in products containing reduced NaCl (1%). The efficacy of organic acids salts in inhibiting *C. perfringens* spore germination and outgrowth was enhanced by sodium nitrite (100 ppm) in the ham formulation. However, *C. perfringens* spore germination and outgrowth was increased if the sample was cooked and chilled after longer storage periods at 5°C. After 24 h of storage, concentrations as high as 2.5% of *Opti.Form* PD4® are needed to control *C. perfringens* outgrowth in uncured ham at 1 or 2 % salt concentration. After 3 h of packaging, inhibition of *C. perfringens* outgrowth occurs in all the treatments tested, including the control samples. The presence of this inhibitory phenomenon can lead to inconsistencies and inappropriate conclusions regarding the role of antimicrobials in meat products.

8.6 References

- Abou-Zeid K. A., Yoon K. S., Oscar T. P., Schwarz J. G., Hashem F. M. and Whiting R. C. 2007. Survival and growth of *Listeria monocytogenes* in broth as a function of temperature, pH and potassium lactate and sodium diacetate concentrations. J. Food Prot. 70: 2620-2625.
- Amezquita A., Weller C. L., Wang L, Thippareddi H. and Burson D. 2005. Development of an integrated model for heat transfer and dynamic growth of *Clostridium perfringens* during the cooling of cooked boneless ham. Int. J. Food Microb. 101: 123-144.
- Apostolidis P., Kwon Y. I. and Shetty K. 2008. Inhibition of *Listeria monocytogenes* by oregano, cranberry and sodium lactate combination in broth and cooked ground beef systems and likely mode of action through proline metabolism. Int. J. Food Microb. 128: 317-324.
- Baros J. A. 2001. Halophilic and Osmophilic Microorganisms. Compendium for methods for the microbiological examination of foods (4th ed.). Pouch F. Washington D.C., American Public Health Association. Pages 187-190.
- Christiansen L. N., Johnston R. W., Kautter D. A., Howard J. W. and Aunan W. J. 1973. Effect of nitrite and nitrate on toxin production by *Clostridium botulinum* and on nitrosamine formation in perishable canned comminuted cured meat. Appl. Microb. 25: 357-362.
- Desmond E. 2006. Reducing salt: A challenge for the meat industry. Meat Science. 74: 188-196.
- Devlieghere F., Vermeiren L., Bontenbal E. Lamers P. P. and Debevere J. 2009. Reducing salt intake from meat products by combined use of lactate and diacetate salts without affecting microbial stability. Int. J. Food Sci. & Tech. 44: 337-341.
- He F. J. and McGregor G. A. 2008. A comprehensive review on salt and health and current experience of worldwide salt reduction programmes. J. of Human Hypert. 1-22.
- Hill, L.H., Webb, N.B., Mongol, L.D. and Adams, A.T. 1973. Changes in residual nitrite in sausages and luncheon meat products during storage. J. Milk Food Technol. 36: 515-519.
- Honikel K. 2008. The use and control of nitrate and nitrite for the processing of meat products. Meat Science. 78: 68-76.
- Huang L. 2003. Dynamic computer simulation of *Clostridium perfringens* growth in cooked ground beef. Int. J. Food Microb. 87: 217-227.
- Jackson A. I., Sullivan G. A., Kulchaiyawat C., Sebranek J. G. and Dickson J. 2011. Survival and growth of *Clostridium perfringens* in commercial no-nitrate-or-nitrite-added (natural and organic) frankfurters, hams, and bacon. J. Food Prot. 74: 410-416.

- Juneja V. K. 2003. Predictive model for the combined effect of temperature, sodium lactate and sodium diacetate on the heat resistance of *Listeria monocytogenes* in beef. J. Food Prot. 66: 804-811.
- Juneja V. K. and Thippareddi H. 2004a. Control of *Clostridium perfringens* in a model roast beef by salts of organic acids during chilling. J. Food Safety. 24: 95-108.
- Juneja V. K. and Thippareddi H. 2004b. Inhibitory effects of organic acid salts on growth of *Clostridium perfringens* from spore inocula during chilling of marinated ground turkey breast. Int. J. Food Microb. 93: 155-163.
- Juneja V. K., Call J. E. and Miller A. J. 1993. Evaluation of methylxanthines and related compounds to enhance *Clostridium perfringens* sporulation using Duncan and Strong medium. J. Rapid Methods Automat. Microbiol. 2: 203-218.
- Juneja V. K., Huang L. and Thippareddi H. 2006. Predictive model for growth of *Clostridium perfringens* in cooked cured pork. Int. J. Food Microb. 110: 85-92.
- Li J. and McClane B. 2006. Comparative effects of osmotic, sodium nitrite induced, and pH induced stress on growth and survival of *Clostridium perfringens* Type A isolates carrying chromosomal or plasmid-borne enterotoxin genes. Appl. And Env. Microb. 72: 7620-7625.
- Lindstrom M., Heikinheimo A, Lahti P. and Korkeala H. 2011. Novel insights into the epidemiology of *Clostridium perfringens* type A poisoning. Food Microbiology. 28: 192-198.
- Mackay J. and Mensah G. 2004. The Atlas of Heart Disease and Stroke. United Kingdom, Myriad Editions.
- McClure P. 2002. Foodborne pathogens: hazards, risks analysis and control. Cambridge, Woodhead Publishing. Page 423.
- Moller J., Jensen J., Olsen M., Skibsted L. and Bertelsen G. 2000. Effect of residual oxygen on colour stability during chill storage of sliced, pasteurized ham packaged in modified atmosphere. Meat Science. 54: 399-405.
- O'Leary V. and Solberg M. 1976. Effect of sodium nitrite inhibition on intracellular thiol groups and on the activity of certain glycolytic enzymes in *Clostridium perfringens*. Appl. and Env. Microb. 31: 208-212.
- Poovey K.A., Burson D. E., Mandigo R. W., Cuppett S. L. and Thippareddi H. 2008. Effect of organic acid salts on quality characteristics of whole muscle hams. Nebraska Swine Reports. Animal Science Department. University of Nebraska, Lincoln.
- Ryan K. and Ray C. 2010. Sherris Medical Microbiology (5ed). New York, McGraw Hill. Pages 520-521.
- Sabah J., Thippareddi H., Marsden J.L. and Fung D. 2003. Use of organic acids for the control of *Clostridium perfringens* in cooked vacuum-packaged restructured roast beef during an alternative cooling procedure. J. Food Prot. 66:1408-1412.
- Shoemaker S. P. and Pierson M. D. 1976. "Phoenix phenomenon" in the growth of *Clostridium perfringens*. Appl. Env. Microb. 32: 803-807.

- Sigrid B. and Granum P. 2002. *Clostridium perfringens* and foodborne infections. Int. J. Food Mic. 74: 195-202.
- Singh A., Korasapati N. R., Juneja V. K. and Thippareddi H. 2010. Effect of phosphate meat (pork) types on the germination and outgrowth of *Clostridium perfringens* spores during abusive chilling. J. Food Prot. 73: 879-887.
- Stringer S. C. and Pin C. 2005. Microbial risks associated with salt reduction in certain foods and alternative options for preservation. Technical report. United Kingdom, Institute of Food Research.
- Taormina P. T. 2010. Implications of salt and sodium reduction on microbial food safety. Crit. Rev. Food Sc. Nutr. 50: 209-227.
- Theron M. M. and Rykers J. F. 2011. Organic acids and food preservation. Boca Raton, Florida, CRC Press.
- Thippareddi H., Juneja V.K., Phebus R., Marsden J.L. and Kastner C.L. 2003. Control of *Clostridium perfringens* germination and outgrowth by buffered sodium citrate during chilling of roast beef and injected pork. J. Food Prot. 66:376-381.
- Tompkin R. B. 2005. Antimicrobials in Food. Davidson P. M., Sofos J. N. and Branen A (Eds.). L. Florida, Taylor & Francis Group. Page 170-171.
- USDA-FSIS. 1999. Performance standards for the production of certain meat and poultry products. FSIS Directive 7111.1 Federal Register 64:732-749.
- USDA-FSIS. 2000. Food additives for use in meat and poultry products: sodium diacetate, sodium acetate, sodium lactate and potassium lactate. Code of Federal Regulations, 9 CFR Part 424 65:3121-3123.
- USDA-AMS. 2010. Institutional Meat Purchase Specifications. Fresh Pork. Series 400. Page 10.
- Vasseur C., Baverel L., Hebraud M. and Labadie J. 1999. Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes*. J. Appl. Microb. 86: 469-476.
- Velugoti P.R., Bohra L. K., Juneja V. J. and Thippareddi H. 2007. Inhibition of germination and outgrowth of *Clostridium perfringens* by lactic acid salts during cooling of injected turkey. J. Food Prot. 70: 923-929.
- Zaika L. L. 2003. Influence of NaCl content and cooling rate on outgrowth of *Clostridium perfringens* spores in cooked ham and beef. J. Food Prot. 66: 1599-1603.

8.7 List of Tables

Table 3.1. Mean pH and water activity values of ham containing various concentrations of sodium chloride, sodium nitrite and *Opti.Form* PD4®.

Treatment			pH ^a	Water activity
NaCl (%)	NaNO ₂ (ppm)	<i>Opti.Form</i> PD4® (%)		
1	-	-	5.69 ± 0.07 ^b	0.994 ± 0.002 ^b
1	-	1.5	5.73 ± 0.02 ^c	0.992 ± 0.004 ^{b,c}
1	-	2.5	5.73 ± 0.01 ^{b,c}	0.986 ± 0.005 ^{b,c,d}
2	-	-	5.81 ± 0.13 ^{b,c}	0.992 ± 0.004 ^{c,e,g,h,k}
2	-	1.5	5.73 ± 0.03 ^{b,c}	0.986 ± 0.003 ^{c,f,g,h,k}
2	-	2.5	5.71 ± 0.01 ^{b,c}	0.985 ± 0.008 ^{d,g,h,j,k}
1	100	-	5.81 ± 0.10 ^c	0.994 ± 0.003 ^b
1	100	1.5	5.77 ± 0.05 ^{b,c}	0.989 ± 0.002 ^{b,g}
1	100	2.5	5.82 ± 0.07 ^{b,c}	0.988 ± 0.005 ^{b,h}
2	100	-	5.75 ± 0.07 ^{b,c}	0.988 ± 0.005 ^{c,f,g,h,k}
2	100	1.5	5.78 ± 0.09 ^c	0.984 ± 0.003 ^{b,k}
2	100	2.5	5.77 ± 0.07 ^{b,c}	0.979 ± 0.003 ^{e,f,j}

^a Mean ± standard deviations; values represent three independent replications and triplicate measurements for each sample.

^b Values followed by the same letter are not statistically different.

8.8 Legend to the Figures

- Fig. 3.1. Mean *Clostridium perfringens* populations (log CFU/g) in ham containing various concentrations of NaCl, NaNO₂ and *Opti. Form* PD4®, prepared and processed within 3 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 9 h (Chill).
- Fig. 3.2. Mean *Clostridium perfringens* populations (log CFU/g) in ham containing various concentrations of NaCl, NaNO₂ and *Opti. Form* PD4®, prepared and processed within 3 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 15 h (Chill).
- Fig. 3.3. Mean *Clostridium perfringens* populations (log CFU/g) in ham containing various concentrations of NaCl, NaNO₂ and *Opti. Form* PD4®, prepared and processed within 3 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 21 h (Chill).
- Fig. 3.4. Mean *Clostridium perfringens* populations (log CFU/g) in ham containing various concentrations of NaCl, NaNO₂ and *Opti. Form* PD4®, prepared and processed within 24 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 15 h (Chill).
- Fig. 3.5. Mean *Clostridium perfringens* populations (log CFU/g) in ham containing various concentrations of NaCl, NaNO₂ and *Opti. Form* PD4®, prepared and processed within 24 h, immediately after heat shock at 75°C for 20 min (Heat Shock), and after cooling exponentially from 54.4°C to 4°C in 21 h (Chill).

Fig. 3.1.

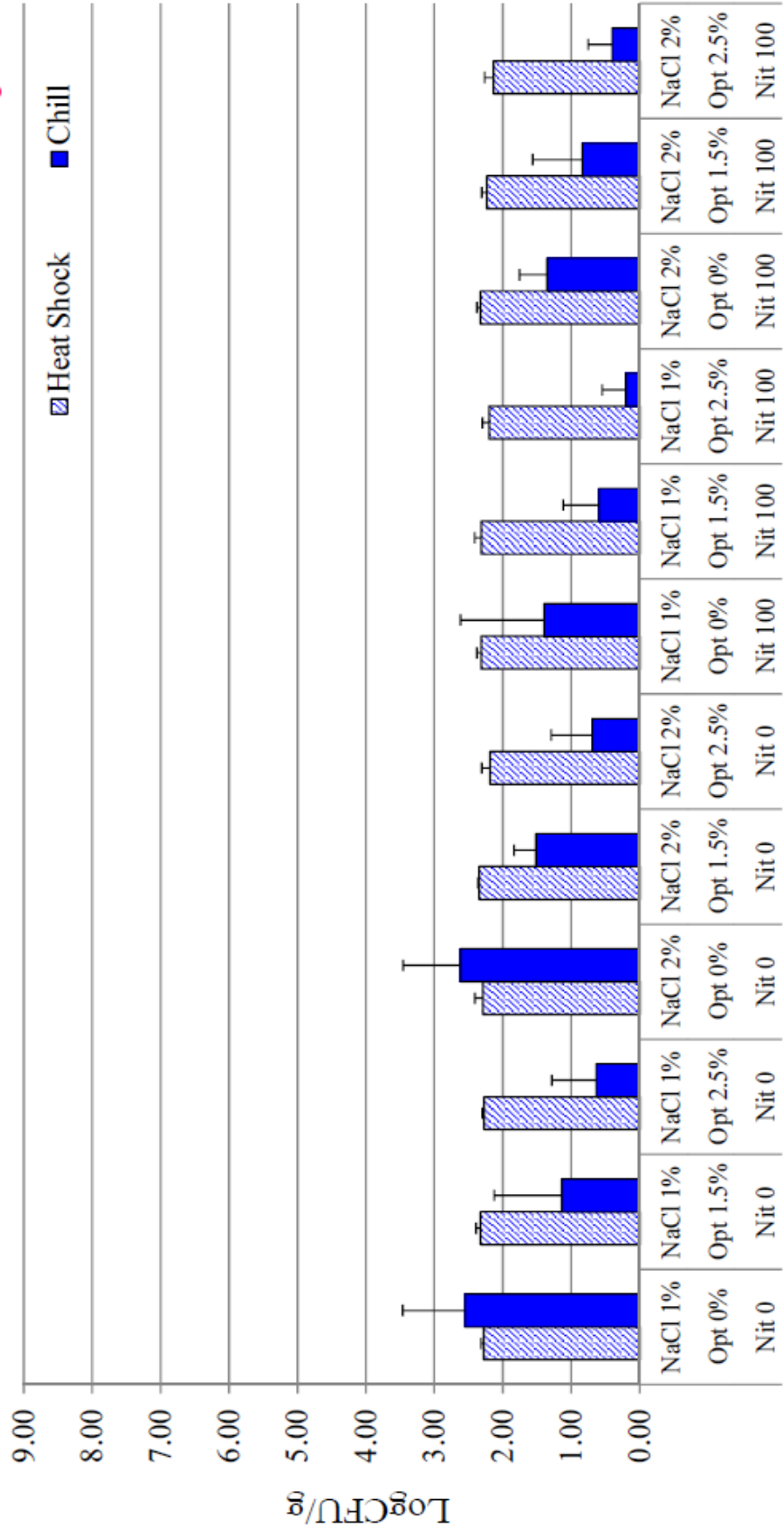


Fig. 3.2.

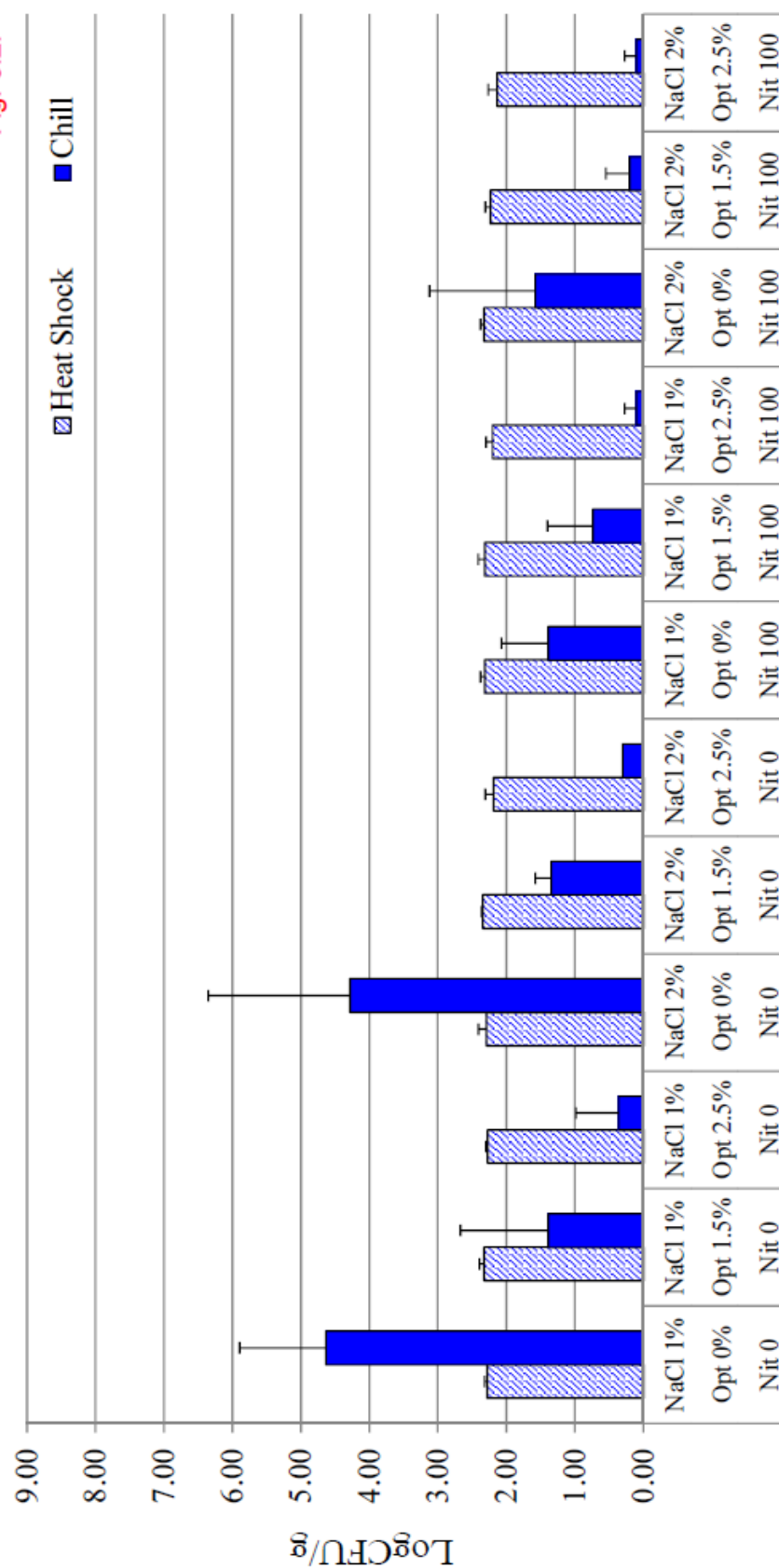


Fig. 3.3.

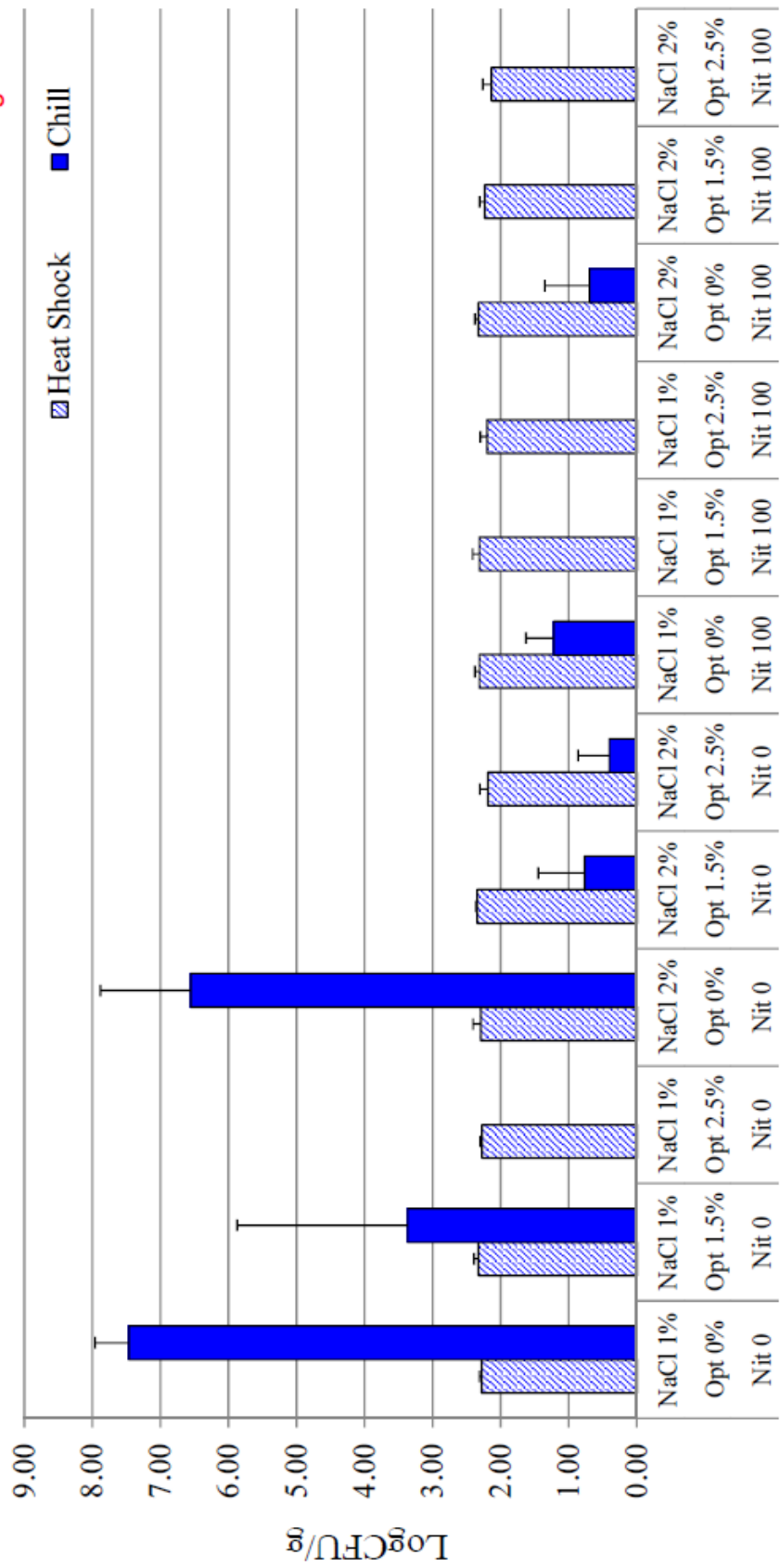


Fig. 3.4.

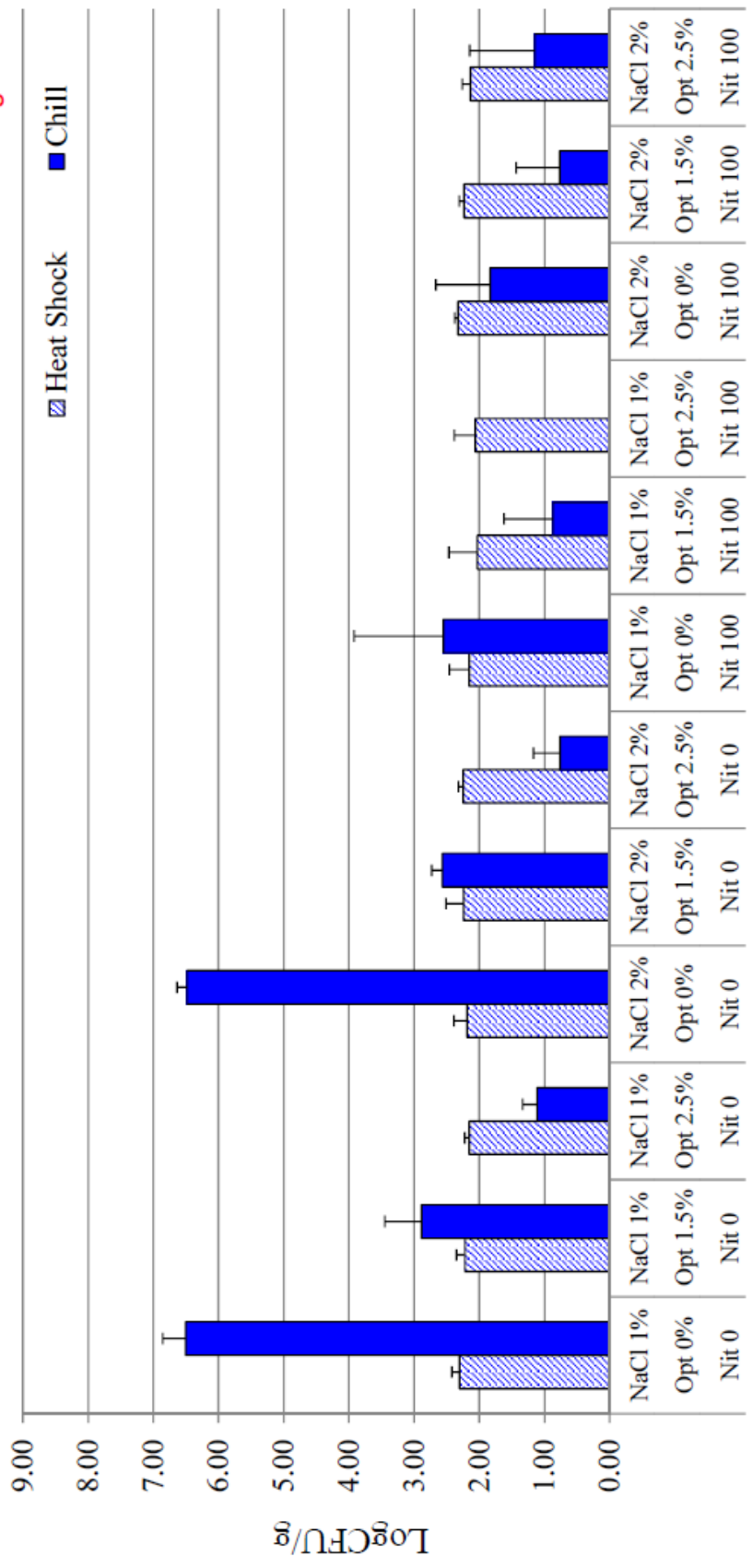
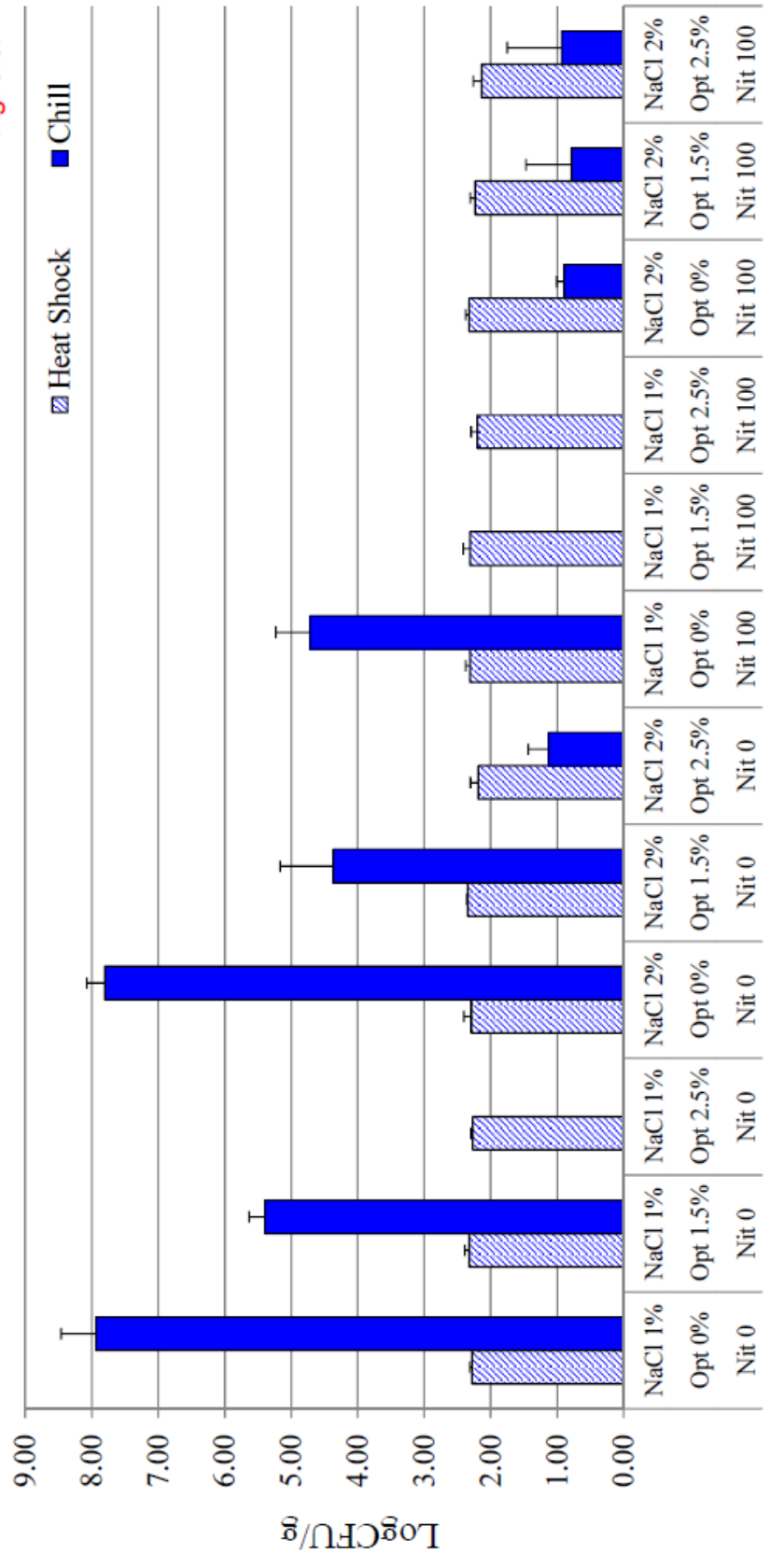


Fig. 3.5.



CHAPTER 4

RECOMMENDATIONS FOR FUTURE RESEARCH

This research evaluated the impact that new meat formulations with reduced NaNO₂ and NaCl have on microbial stability, specifically, on *C. perfringens* spore germination and outgrowth. Concentration of the ingredients and processing conditions have an impact on *C. perfringens* spore germination and outgrowth during abusive cooling. Future research should be directed to clarify some important aspects:

1. The role of residual oxygen in *C. perfringens* inhibition should be confirmed. Experiments could be designed in order to address the oxidative processes occurring in the meat system and how they inhibit *C. perfringens* spore germination and outgrowth. Aspects to be evaluated can include, time of packaging, sample size, effect of formulation, effect of storage temperature, effect of meat type and duration of the inhibition.
2. Antimicrobial activity of sodium erythorbate must be confirmed. Recommendation includes the design of experiments to identify which processing conditions affect antimicrobial activity of sodium erythorbate including temperature and storage time. Future experiments should be performed to address the relationship between erythorbate, residual nitrite and microbial stability of the product.
3. Quality effects of new product formulations should be assessed. Research should couple quality analysis with microbial studies. Sensory attributes of new formulations must be evaluated, especially in products formulated with reduced NaCl levels and organic acid salts. Recommendations can be made for meat processors if these aspects are evaluated simultaneously.

APPENDIX

A.1. Mean *C. perfringens* populations (\pm SD) in ham formulated with different concentrations of NaNO₂ and cooled from 54.4°C to 4.4°C within 15 h subsequent to 3 h of storage at 5°C after preparation.

Treatment		Initial counts (LogCFU/g)	Final counts (LogCFU/g)
NaNO ₂	Sodium erythorbate		
-	-	2.31 \pm 0.11	3.76 \pm 0.66
50	-	2.25 \pm 0.07	2.83 \pm 0.78
100	-	2.16 \pm 0.30	1.61 \pm 1.16
150	-	2.03 \pm 0.44	2.00 \pm 0.95
200	-	2.06 \pm 0.32	0.81 \pm 0.93
50	557	2.22 \pm 0.13	5.22 \pm 0.81
100	557	2.15 \pm 0.07	3.34 \pm 0.61
150	557	2.19 \pm 0.20	1.16 \pm 0.76
200	557	2.24 \pm 0.27	0.71 \pm 0.77

A.2. Mean *C. perfringens* populations (\pm SD) in ham formulated with different concentrations of NaNO₂ and cooled from 54.4°C to 4.4°C within 15 h subsequent to 24 h of storage at 5°C after preparation.

Treatment		Initial counts (LogCFU/g)	Final counts (LogCFU/g)
NaNO ₂	Sodium erythorbate		
-	-	2.30 \pm 0.11	6.50 \pm 0.49
50	-	2.15 \pm 0.15	5.49 \pm 1.31
100	-	2.15 \pm 0.12	5.16 \pm 0.97
150	-	2.19 \pm 0.08	4.41 \pm 0.55
200	-	2.04 \pm 0.10	2.34 \pm 0.54
50	557	2.23 \pm 0.27	6.94 \pm 0.69
100	557	2.24 \pm 0.22	5.96 \pm 0.82
150	557	2.20 \pm 0.20	2.88 \pm 0.98
200	557	2.25 \pm 0.14	1.68 \pm 0.14

A.3. Mean *C. perfringens* populations (\pm SD) in ham formulated with different concentrations of NaCl and cooled from 54.4°C to 4.4°C within 9 h subsequent to 3 h of storage at 5°C after preparation.

Treatment			Initial counts	Final counts
NaCl (%)	NaNO ₂ (ppm)	<i>Opti.Form</i> PD4® (%)	(LogCFU/g)	(LogCFU/g)
1.0	-	-	2.28 \pm 0.03	2.55 \pm 0.91
1.0	-	1.5	2.32 \pm 0.07	1.14 \pm 0.99
1.0	-	2.5	2.27 \pm 0.03	0.63 \pm 0.65
2.0	-	-	2.29 \pm 0.11	2.62 \pm 0.84
2.0	-	1.5	2.35 \pm 0.01	1.51 \pm 0.32
2.0	-	2.5	2.19 \pm 0.12	0.69 \pm 0.60
1.0	100	-	2.32 \pm 0.06	1.40 \pm 1.22
1.0	100	1.5	2.32 \pm 0.10	0.59 \pm 0.53
1.0	100	2.5	2.20 \pm 0.09	0.20 \pm 0.35
2.0	100	-	2.33 \pm 0.05	1.35 \pm 0.40
2.0	100	1.5	2.23 \pm 0.08	0.84 \pm 0.72
2.0	100	2.5	2.14 \pm 0.13	0.40 \pm 0.35

A.4. Mean *C. perfringens* populations (\pm SD) in ham formulated with different concentrations of NaCl and cooled from 54.4°C to 4.4°C within 15 h subsequent to 3 h of storage at 5°C after preparation.

Treatment			Initial counts	Final counts
NaCl (%)	NaNO ₂ (ppm)	<i>Opti.Form</i> PD4® (%)	(LogCFU/g)	(LogCFU/g)
1.0	-	-	2.28 \pm 0.03	4.63 \pm 1.26
1.0	-	1.5	2.32 \pm 0.07	1.38 \pm 1.28
1.0	-	2.5	2.27 \pm 0.03	0.36 \pm 0.62
2.0	-	-	2.29 \pm 0.11	4.28 \pm 2.07
2.0	-	1.5	2.35 \pm 0.01	1.34 \pm 0.23
2.0	-	2.5	2.19 \pm 0.12	0.30 \pm 0.00
1.0	100	-	2.32 \pm 0.06	1.39 \pm 0.68
1.0	100	1.5	2.32 \pm 0.10	0.73 \pm 0.67
1.0	100	2.5	2.20 \pm 0.09	0.10 \pm 0.17
2.0	100	-	2.33 \pm 0.05	1.58 \pm 1.55
2.0	100	1.5	2.23 \pm 0.08	0.20 \pm 0.35
2.0	100	2.5	2.14 \pm 0.13	0.10 \pm 0.17

A.5. Mean *C. perfringens* populations (\pm SD) in ham formulated with different concentrations of NaCl and cooled from 54.4°C to 4.4°C within 21 h subsequent to 3 h of storage at 5°C after preparation.

Treatment			Initial counts	Final counts
NaCl (%)	NaNO ₂ (ppm)	<i>Opti.Form</i> PD4® (%)	(LogCFU/g)	(LogCFU/g)
1.0	-	-	2.28 \pm 0.03	7.46 \pm 0.50
1.0	-	1.5	2.32 \pm 0.07	3.37 \pm 2.51
1.0	-	2.5	2.27 \pm 0.03	0.00 \pm 0.00
2.0	-	-	2.29 \pm 0.11	6.55 \pm 1.33
2.0	-	1.5	2.35 \pm 0.01	0.77 \pm 0.68
2.0	-	2.5	2.19 \pm 0.12	0.40 \pm 0.46
1.0	100	-	2.32 \pm 0.06	1.22 \pm 0.41
1.0	100	1.5	2.32 \pm 0.10	0.00 \pm 0.00
1.0	100	2.5	2.20 \pm 0.09	0.00 \pm 0.00
2.0	100	-	2.33 \pm 0.05	0.69 \pm 0.65
2.0	100	1.5	2.23 \pm 0.08	0.00 \pm 0.00
2.0	100	2.5	2.14 \pm 0.13	0.00 \pm 0.00

A.6. Mean *C. perfringens* populations (\pm SD) in ham formulated with different concentrations of NaCl and cooled from 54.4°C to 4.4°C within 9 h subsequent to 24 h of storage at 5°C after preparation.

Treatment			Initial counts	Final counts
NaCl (%)	NaNO ₂ (ppm)	<i>Opti.Form</i> PD4® (%)	(LogCFU/g)	(LogCFU/g)
1.0	-	-	2.30 \pm 0.11	3.96 \pm 0.63
1.0	-	1.5	2.23 \pm 0.27	2.40 \pm 0.24
1.0	-	2.5	2.24 \pm 0.22	1.57 \pm 0.05
2.0	-	-	2.20 \pm 0.20	3.63 \pm 0.37
2.0	-	1.5	2.25 \pm 0.14	2.03 \pm 0.10
2.0	-	2.5	2.15 \pm 0.15	1.74 \pm 0.10
1.0	100	-	2.15 \pm 0.12	1.82 \pm 0.09
1.0	100	1.5	2.19 \pm 0.08	1.35 \pm 0.13
1.0	100	2.5	2.04 \pm 0.10	0.00 \pm 0.00
2.0	100	-	2.33 \pm 0.05	1.57 \pm 0.08
2.0	100	1.5	2.23 \pm 0.08	1.21 \pm 0.58
2.0	100	2.5	2.14 \pm 0.13	1.07 \pm 0.01

A.7. Mean *C. perfringens* populations (\pm SD) in ham formulated with different concentrations of NaCl and cooled from 54.4°C to 4.4°C within 15 h subsequent to 24 h of storage at 5°C after preparation.

Treatment			Initial counts	Final counts
NaCl (%)	NaNO ₂ (ppm)	<i>Opti.Form</i> PD4® (%)	(LogCFU/g)	(LogCFU/g)
1.0	-	-	2.31 \pm 0.11	6.50 \pm 0.35
1.0	-	1.5	2.22 \pm 0.13	2.89 \pm 0.56
1.0	-	2.5	2.15 \pm 0.07	1.12 \pm 0.22
2.0	-	-	2.19 \pm 0.20	6.48 \pm 0.15
2.0	-	1.5	2.24 \pm 0.27	2.56 \pm 0.17
2.0	-	2.5	2.25 \pm 0.07	0.76 \pm 0.41
1.0	100	-	2.16 \pm 0.30	2.55 \pm 1.37
1.0	100	1.5	2.03 \pm 0.44	0.87 \pm 0.75
1.0	100	2.5	2.06 \pm 0.32	0.00 \pm 0.00
2.0	100	-	2.33 \pm 0.05	1.83 \pm 0.83
2.0	100	1.5	2.23 \pm 0.08	0.77 \pm 0.67
2.0	100	2.5	2.14 \pm 0.13	1.15 \pm 1.00

A.8. Mean *C. perfringens* populations (\pm SD) in ham formulated with different concentrations of NaCl and cooled from 54.4°C to 4.4°C within 21 h subsequent to 24 h of storage at 5°C after preparation.

Treatment			Initial counts	Final counts
NaCl (%)	NaNO ₂ (ppm)	<i>Opti.Form</i> PD4® (%)	(LogCFU/g)	(LogCFU/g)
1.0	-	-	2.28 \pm 0.03	7.93 \pm 0.53
1.0	-	1.5	2.32 \pm 0.07	5.39 \pm 0.24
1.0	-	2.5	2.27 \pm 0.03	0.00 \pm 0.00
2.0	-	-	2.29 \pm 0.11	7.80 \pm 0.27
2.0	-	1.5	2.35 \pm 0.01	4.37 \pm 0.80
2.0	-	2.5	2.19 \pm 0.12	1.13 \pm 0.31
1.0	100	-	2.32 \pm 0.06	4.71 \pm 0.52
1.0	100	1.5	2.32 \pm 0.10	0.00 \pm 0.00
1.0	100	2.5	2.20 \pm 0.09	0.00 \pm 0.00
2.0	100	-	2.33 \pm 0.05	0.89 \pm 0.11
2.0	100	1.5	2.23 \pm 0.08	0.79 \pm 0.68
2.0	100	2.5	2.14 \pm 0.13	0.94 \pm 0.82

A.9. Formulation^a for ham (control) prepared to evaluate the effect of different NaNO₂ and sodium erythorbate levels on *C. perfringens* spore germination and outgrowth during abusive cooling (Chapter 2).

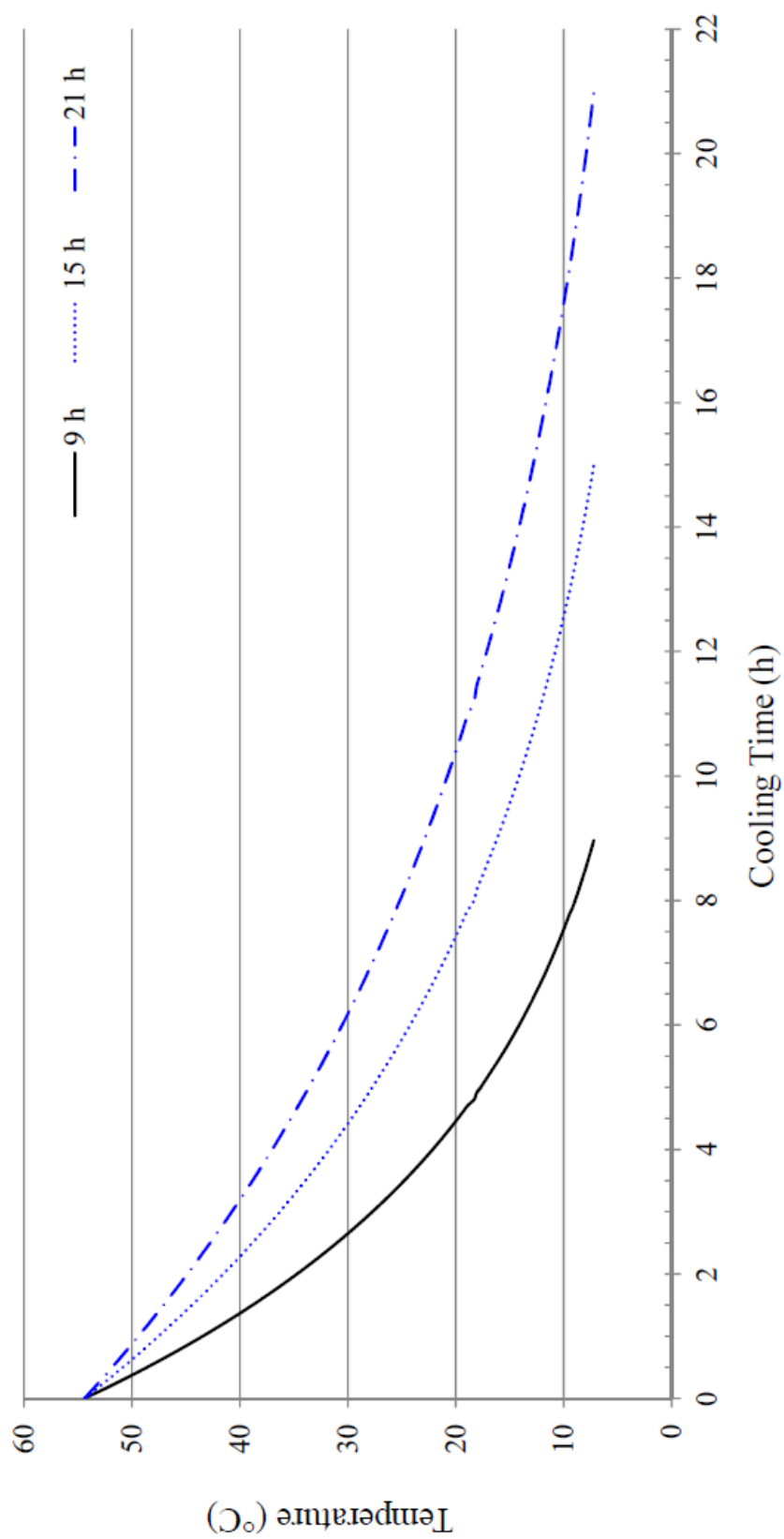
Ingredient	Grams	Concentration
Meat Block	500	100%
NaNO ₂	0.4	50 ppm
Sodium erythorbate	0	0 ppm
Phosphates	1.5	0.3%
Sugar	2.5	0.5%
Salt	9.62	2%
Water	146	29.2%

^a32% pump rate

A.10. Formulation^a for ham (control) prepared to evaluate the effect of different levels of NaNO₂, NaCl and *Opti.Form* PD4® on *C. perfringens* spore germination and outgrowth during abusive cooling (Chapter 3).

Ingredient	Grams	Concentration
Meat Block	500	100%
NaNO ₂	0	0 ppm
Sodium erythorbate	0	0 ppm
Phosphates	1.5	0.3%
Sugar	2.5	0.5%
Salt	5	1%
<i>Opti.Form</i> PD4®	0	0%
Water	150.72	30.14%

^a32% pump rate



A Fig. 1. Temperature profiles for cooling of hams from 54.4°C to 4.4°C within 9, 15 or 21 h.